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UNIVERSITY OF ALBERTA

CARDIAC FUNCTION AND REGULATION OF INTRACELLULAR Ca^{2+} AT LOW TEMPERATURE IN HIBERNATING AND NON-HIBERNATING MAMMALS

BY

BIN LIU ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1993

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ABSTRACT

The effects of low temperature on cardiac cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and different aspects of excitation-contraction coupling have been studied, and compared between a mammalian hibernator (the Richardson's ground squirrel) and a non-hibernator (the rat). $[Ca^{2+}]_i$, measured with fluorescent Indo-1, increases significantly from 139 nM at 37°C to 255 nM at 15°C and 297 nM at 5°C, respectively, in isolated rat ventricular myocytes. However, $[Ca^{2+}]_i$ in isolated ventricular myocytes from ground squirrels is less influenced by low temperature, being 103 nM at 37°C, 138 nM at 15°C, and 168 nM at 5°C. The ability to maintain $[Ca^{2+}]_i$ at low levels at low temperature in ground squirrels is related to activity of the sarcolemmal Na^+-Ca^{2+} exchanger in Ca^{2+} extrusion from the cell, and of Ca^{2+}-ATPase in the sarcoplasmic reticulum (SR) in SR Ca^{2+} uptake.

In addition to a reduced Ca^{2+} influx through L-type Ca^{2+} channels shown in previous studies, the effect of organic Ca^{2+} channel blockers (nifedipine and verapamil) on muscle contraction is decreased at low temperature either due to a direct effect of cooling or due to use-dependency of the blocker. In contrast, the sensitivity of muscle contraction to Cd^{2+} increases at low temperature in both rats and ground squirrels. Whether this is due to its direct effect on Ca^{2+} channels or due to its intracellular effect needs to be further investigated.

When Ca^{2+} sensitivity of myofilaments is examined in chemically-skinned cardiac muscles, a reduced Ca^{2+} sensitivity of the myofilaments at low temperature in both rats and ground squirrels suggests that hypothermia-induced positive inotropy in cardiac muscles is probably due to increased Ca^{2+} release from the SR.

Taken together, the results obtained in the present study are consistent with

the hypothesis that Ca^{2+} overload induced by cooling may be one of the causes of severe arrhythmias in accidental hypothermia. Maintenance of cardiac contractile function at low temperature requires a superior ability to regulate $[Ca^{2+}]_i$. Since abnormal increase of $[Ca^{2+}]_i$ impairs cellular metabolism and induces cell injury, reduction of $[Ca^{2+}]_i$ at low temperature needs to be considered in hypothermic heart preservation. Preliminary data have shown that perfusion with low Ca^{2+} (0.5 mM) before cooling improves functional recovery of the rat heart after perfused preservation at 5°C for 18 hours.

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I. INTRODUCTION

Cold tolerance of cardiac cells is one of the unique properties of mammalian hibernators (Chao and Yeh 1951; Dawe and Morrison 1955; Lyman and Blinks 1959; Johansson 1985; Johansson 1991). Regardless of whether the animal is in natural deep hibernation (Kayser 1961; Lyman et al. 1982; Wang 1988), or in experimental hypothermia (Popovic 1960; Jourdan and Wang 1987; Jourdan 1991), the contractile function of the heart is well-adapted to supply oxygen and substrates to vital tissues, such as the brain, liver and the heart itself.

During natural deep hibernation, the core body temperature (T_b) of field animals can be measured with radiotelemetry (Wang 1973; Wang 1979). T_b is typically 2-8°C (Wang 1988) but can be as low as -2.9°C under supercooling in the Arctic ground squirrels (Barnes 1989). The duration of a single hibernation bout can be from a few days to several weeks (Wang 1988), and can last up to 76 days in bats (Twente et al. 1985). During entrance into hibernation, heart rate decreases gradually, followed by T_b. When deep hibernation is attained (T_b=2-8°C), heart rate decreases to 2-10 beats/min (Dawe and Morrison 1955; Lyman and O'Brien 1960). Systolic pressure varies from 40 to 90 mm Hg and diastolic pressure from 10 to 40 mm Hg (Lyman and O'Brien 1960). A marked increase in peripheral resistance has been observed in hibernating marmots and thirteen-lined ground squirrels (Lyman and O'Brien 1960; Zatzman and Thornhill 1987). However, due to the marked decrease in heart rate, cardiac output is reduced even though the stroke volume may increase. Consequently, the mean arterial pressure of marmots may decrease by ~50% from 100-130 mm Hg in the active state to 50-60 mm Hg in the hibernating state (Zatzman and Thornhill 1987). Using endogenous energy supplies, hibernators can rewarm spontaneously within 4 hours. Heart rate, blood pressure, and T_b are restored to euthermic conditions without any impairment of heart function (Dawe and Morrison 1955; Lyman and

O'Brien 1960; Eagles et al. 1988).

Maintenance of cardiac contractile function at low temperature in hibernators has also been observed in experimental hypothermia. Artificially cooled thirteen-lined ground squirrels have survived 110 hours at a T_b of 10°C (Popovic 1960), while Richardson's ground squirrels have survived 96 hours at a T_b of 7°C (Jourdan 1991). Contractile function of the heart is maintained during cooling, in deep hypothermia, and during rewarming; and changes in heart rate, cardiac output, stroke volume, peripheral resistance, and blood pressure are similar to those seen in hibernation (Sjöquist et al. 1986).

In contrast to hibernators, the limited cold tolerance of the human heart is one of the major concerns in the rescue of victims from accidental hypothermia (Solomon et al. 1989). Clinical reports indicate that during the early stages of hypothermia, along with cutaneous vasoconstriction, a sinus tachycardia develops to support enhanced heat production by shivering. When T_b decreases to about 32°C, there is a progressive inhibition of the sinus rhythm and an ectopic atrial focus develops frequently. Around 30°C, irritability of the atria increases, leading to atrial flutter or fibrillation. Further decrease of T_b leads to increased ventricular excitability, resulting in premature ventricular contractions and ventricular fibrillation. Asystole occurs eventually at about 15°C. During the process of assisted rewarming from hypothermia, a persistent ventricular fibrillation occurs in most cases. A stable sinus rhythm usually cannot be established until T_b is higher than 30°C (Nordrehaug 1982; Husby et al. 1990). Similar problems have also been observed in other non-hibernators in experimental hypothermia (Thauer 1965).

The effects of low temperature on heart function have also been studied in the isolated perfused heart. Heart rate decreases exponentially upon cooling in both hibernators and non-hibernators (Lyman and Blinks 1959). The relationship of heart rate to temperature can be described by the Arrhenius equation (Senturia et al. 1970). In hibernators including bats, golden hamsters, hedgehogs, marmots, and several species of ground squirrels, the isolated heart is able to maintain contractions at 0-7°C (Chao and Yeh 1951; Lyman and Blinks 1959; Michael and Menaker 1963; Senturia et al. 1970; Caprette and Senturia 1984; Jones and Romano 1984). Even without perfusion or oxygenation, the isolated hearts from active thirteen-lined ground squirrels can maintain their contractions at 5°C for up to 3 hours; impressively, this time period can be doubled in hearts isolated from hibernating individuals (Spurrier and Dawe 1977). In contrast, hearts isolated from non-hibernators under Langendorff perfusion become severely arrhythmic at 30-16°C and stop beating at 16-10°C (Lyman and Blinks 1959; Caprette and Senturia 1984; Burlington and Darvish 1988). Coronary flow decreases upon cooling in both hibernators and non-hibernators. The ability to regulate coronary flow is maintained at 7°C in a hibernating species, the thirteen-lined ground squirrel, but lost in a non-hibernating species, the rat (Burlington et al. 1989).

Since energy supply is essential to contractile function of the heart, the energy reserves of the isolated heart at low temperatures have been compared between hibernators and non-hibernators. Although the concentrations of ATP and creatine phosphate are decreased at 12-6°C in thirteen-lined ground squirrels, the phosphorylation potential, i.e. $ATPx10^3/ADPxP_i$, is maintained at the control level observed at 35°C (Burlington et al. 1976). This indicates that the energy status is well-balanced among the processes of ATP synthesis, translocation, and utilization in the cardiac cells of hibernators. ATP is not depleted in rat hearts at 12-6°C; however, the phosphorylation potential decreases markedly at these temperatures. Therefore, an imbalance of ATP synthesis, translocation, and utilization may account in part for the cardiac failure observed during hypothermia (Burlington et al. 1976).

The kinetics of cardiac contractions at low temperatures has been studied in isolated ventricular papillary muscles or muscle strips (Blinks and Koch-Weser 1963). In non-hibernators, including rats, rabbits and guinea pigs, an increase in the magnitude of muscle contraction under electrical stimulation has been observed when temperature is decreased from 37°C to 25-20°C (Smith and Katzung 1966; Langer and Brady 1968; South and Jacobs 1973; Mattiazzi and Nilsson 1976; Liu et al. 1990). Further decreases in temperature result in reduction of muscle contraction. In hibernators, a similar low temperature-induced inotropy is also evident in golden hamsters, hedgehogs, and several species of ground squirrels as temperature is decreased from 37°C to 25-20°C (Smith and Katzung 1968; Alpert et al. 1972; South and Jacobs 1973; Zhou et al. 1987; Liu et al. 1990; Zhou et al. 1991). However, in contrast to non-hibernators, the magnitude of contraction continues to increase when temperature is further decreased to 15-10°C. In hedgehog ventricular muscles, the magnitude of contraction remains at a relatively high level even at 5-0°C (Liu et al. 1990). The maximal rate of force production ($+dF/dt_{max}$) decreases upon cooling in rats, golden hamsters and hedgehogs. But a biphasic change in $+dF/dt_{max}$ is observed in both a non-hibernator, the rabbit (Liu et al. 1990) and a hibernator, the Richardson's ground squirrel (Wang 1988): an increase in $+dF/dt_{max}$ between 37 and 20°C, followed by a marked decrease below 20°C. The time to peak force of muscle contraction increases exponentially at low temperature in both hibernators, hedgehogs and Richardson's ground squirrels (Liu et al. 1990; Wang 1988), and non-hibernators, rats and rabbits (Liu et al. 1990). In addition, the time from peak force to half-relaxation (THR) also increases exponentially upon cooling. Although no significant difference in THR is observed between the hedgehogs and two non-hibernators (rats and rabbits), the increase of THR with decreasing temperature is always less in the hedgehogs between 25 and 10°C, suggesting that the processes governing muscle relaxation are less influenced by low temperature in the hibernator.

In addition to the kinetic analysis of muscle contraction, the resting tension (T_{rest}) of isolated ventricular muscles at low temperature has also been compared between a hibernator, the hedgehog and two non-hibernators, the rat and rabbit (Liu et al. 1990). In the ventricular muscle of rats and rabbits, the T_{rest} increases markedly below 20-15°C. Arrhythmic aftercontractions occur when temperature is further decreased. In the hedgehog, on the other hand, lowering the temperature has no effect on T_{rest}, and the electrically stimulated muscle continues to contract smoothly even at 0°C. Since the changes in T_{rest} correlate closely to cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) (Sheu and Fozzard 1982) and aftercontractions have been observed as a consequence of an abnormal increase in $[Ca^{2+}]_i$ (Jensen and Katzung 1968; Allen et al. 1985), it has been proposed that the cardiac cells of non-hibernators become Ca^{2+}-overloaded at low temperature (Liu et al. 1990). This might be the main cause of severe cardiac arrhythmia observed in deep hypothermia. However, no direct measurements of cardiac $[Ca^{2+}]_i$ at different temperatures have been reported, nor has there been any documentation on the regulation of $[Ca^{2+}]_i$ and cardiac muscle contraction at low temperature.

In mammalian cardiac cells, contractile function is based on a series of cellular events underlying excitation-contraction coupling (Langer 1990; Bers 1991; Callewaert 1992; Stern and Lakatta 1992). Depolarization of the sarcolemmal membrane induces a Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Recent studies also suggest that Ca^{2+} can enter into the cell via a sarcolemmal Na^+-Ca^{2+} exchange mechanism upon membrane depolarization. The major function of this Ca^{2+} influx is to trigger Ca^{2+} release from intracellular Ca^{2+} stores, the sarcoplasmic reticulum (SR). The resulting rapid increase in $[Ca^{2+}]_i$ activates the myofilaments and initiates mechanical contraction. Ca^{2+} uptake back into the SR via SR Ca^{2+}-ATPase, and Ca^{2+} extrusion via the sarcolemmal Na^+-Ca^{2+} exchanger and Ca^{2+}-ATPase reduce $[Ca^{2+}]_i$ and cause muscle relaxation.

Comparative studies have shown that the effects of low temperature on cardiac excitation-contraction coupling are markedly different between mammalian hibernators and non-hibernators. For instance, in the cardiac cells of two hibernators (the hedgehog and thirteen-lined ground squirrel), the sarcolemmal membrane is still excitable below 10°C (Marshall and Willis 1962; Liu et al. 1987; Svensson et al. 1988). The resting membrane potential is in the range of -60 to -50 mV and the transmembrane action potential can be recorded even at 0-6°C. Since Na^+ channels are inactivated when the resting membrane potential is more positive than -60 mV, it has been proposed that the activation of Ca^{2+} channels may be responsible for the excitation of the sarcolemmal membrane at 5°C, a typical T_b in mammalian hibernation (Liu et al. 1987). This has been confirmed in ventricular muscle of summer active hedgehogs in which the action potential is totally abolished in Ca^{2+}-free solution at 5°C (Liu et al. 1987). In voltage-clamp experiments, the Ca^{2+} influx (I_{Ca}) through the voltage-dependent Ca^{2+} channels is measured at different temperatures. The peak I_{Ca} of hedgehog cardiac cells does not change significantly upon cooling, but the inactivation of I_{Ca} is slowed when temperature is decreased from 35 to 10°C (Liu et al. 1991). In contrast, the peak I_{Ca} decreases in single ventricular myocytes of the Richardson's ground squirrels when temperature is decreased from 36 to 12°C (Herve et al. 1992). This suggests a species difference in temperature effects on Ca^{2+} influx amongst hibernators.

In non-hibernators, the sarcolemmal membrane depolarizes markedly below 20°C (Webb et al. 1969). The resting potential becomes more positive than -40 mV at 10°C (Webb et al. 1969; Chapman 1986) and the action potential cannot be recorded below this temperature. The inactivation curve of the Na^+ current shifts to more negative potentials under cooling in guinea pig myocytes (Murray et al. 1990), rat papillary muscles (Liu et al. 1991), and sheep (Dudel and Rudel 1970) and rabbit (Colatsky 1980) Purkinje fibers. This means that the Na^+ channels are no longer available for membrane excitation below 10°C. A reduction of peak I_{Ca} by cooling has also been observed in single myocytes from rats (Mitchell et al. 1983) and guinea pigs (Cavalie et al. 1985, Herve et al. 1992). It seems therefore that dysfunction of both Na^+ and Ca^{2+} channels at low temperature is responsible for failure to elicit membrane depolarization and excitation-contraction coupling in non-hibernators. In addition to the above changes, a transient inward current (I_{TI}) has been observed in rat ventricular cells at 20°C, the magnitude of which becomes more pronounced when temperature is further decreased (Liu et al. 1991). In contrast, this I_{TI} is not observed in the hibernator, the European hedgehog, even at 10°C. Since the I_{TI} is induced an abnormal increase in $[Ca^{2+}]_i$, these results further suggest an impaired cellular Ca^{2+} regulation at low temperature in non-hibernators.

The function of cardiac SR in Ca^{2+} uptake has also been investigated at low temperature. When the rate of SR Ca^{2+} uptake at different temperatures is evaluated with the Arrhenius plot, a linear relationship is seen in two non-hibernators (the dog and rabbit) between 37 and 10°C (Suko 1973); but a curvilinear relationship is seen in a hibernator (the Richardson's ground squirrel) between 37 and 5°C (Belke et al. 1987). These studies suggest a differential effect of low temperature on the SR Ca^{2+} uptake between hibernators and non-hibernators. However, the differences observed in the two studies may be due to the different techniques employed: one with the Millipore filtration of ^{45}Ca at pH 7.0 (Suko 1973) and the other with Ca^{2+}-electrode at pH 7.385 (Belke et al. 1987). Therefore, changes in SR Ca^{2+} uptake and SR Ca^{2+}-ATPase activity at low temperature in hibernators and non-hibernators need to be further studied.

Since mammalian hibernators experience T_b's from 37 to -2.9°C in shifting between the active and the hibernating states, they provide a unique model for understanding how natural selection has permitted cardiac cell survival and maintenance of functional integrity at depressed T_b in this group of animals but not in non-hibernating species. Understanding the mechanisms which confer cold tolerance in cardiac cells in hibernators is also relevant to the advancement of clinical heart transplantation. One of the most critical problems in heart transplantation is the availability of donor hearts and the maintenance of their patency (Burdine et al. 1990; Havel et al. 1991). In order to reduce cellular metabolism and spare energy reserves, the donor heart is usually preserved at low temperature (4-0°C) prior to transplantation (Webb 1969; Wheeldon et al. 1992). Unfortunately, heart function deteriorates with time under hypothermic preservation and only 4-6 hours of preservation time are available before transplantation (Thomas et al. 1978; Billingham et al. 1980; Molina et al. 1985). In some experimental animals (e.g. dog and rabbit), the hypothermic preservation can be extended to 24 hours (Swanson et al. 1988; Wicomb and Collins 1989; Stringham et al. 1992). Since a single episode of hibernation may last several weeks with the heart fully functional at 0-5°C, comparative studies of cardiac function in the hibernators may provide valuable insight as to how the duration of hypothermic heart preservation may be prolonged. This would increase the donor pool and provide more time for long distance transportation, tissue typing, and preparation of the recipient for operating procedures.

Taken together, electrophysiological and mechanical studies have suggested impaired cellular Ca^{2+} homeostasis at low temperature in cardiac cells of non-hibernators but not in hibernators. The aim of this thesis is therefore to compare, between the two groups, the cardiac $[Ca^{2+}]_i$ and the regulation of $[Ca^{2+}]_i$ in relation to muscle contraction at low temperature. Since the effect of low temperature on cardiac Ca^{2+} influx through the voltage-dependent Ca^{2+} channels has already been studied in both hibernating (Liu et al. 1991; Herve et al. 1992) and non-hibernating species (Mitchell et al 1983; Cavalie et al. 1985; Liu et al. 1991), the focus of this thesis is on other aspects of cardiac excitation-contraction coupling as affected by low temperature, including Ca^{2+} uptake by SR, activity of sarcolemmal Na^+-Ca^{2+} exchanger, and Ca^{2+} sensitivity of myofilaments. As a potential application to biomedicine, knowledge obtained from the present study on $[Ca^{2+}]_i$ regulation at low temperature has also been applied to test if improved hypothermic preservation of hearts from a non-hibernator (e.g. rat) can indeed be accomplished.

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II. CYTOSOLIC FREE Ca^{2+} IN RAT VENTRICULAR MYOCYTES AT DIFFERENT TEMPERATURES [1]

INTRODUCTION

Regulation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is important to the function of cardiac cells. During excitation-contraction coupling, Ca^{2+} enters cells through the voltage-dependent Ca^{2+} channels (Tsien 1983) as well as a Na^+-Ca^{2+} exchanger (Beuckelmann and Wier 1989). A major part of the Ca^{2+} influx triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) (Fabiato 1986); however some of the Ca^{2+} influx may directly initiate mechanical contractions (Rich et al. 1988). To restore the low $[Ca^{2+}]_i$ characteristic of the resting state, released Ca^{2+} must be sequestered by the SR and the excess Ca^{2+} extruded out of the cell by the Na^+-Ca^{2+} exchanger (Powell and Noble 1989) and the Ca^{2+}-ATPase in the sarcolemmal membrane (Carafoli 1990). Any disturbance leading to an abnormal increase in $[Ca^{2+}]_i$ will usually result in arrhythmic contractions (Allen and Orchard 1987). The development of new $[Ca^{2+}]_i$ indicators, such as Fura-2 and Indo-1, has allowed more accurate measurements of both resting $[Ca^{2+}]_i$ and Ca^{2+} transient during excitation-contraction coupling not only in normal physiological conditions, but also during ischemia and under inhibition of cellular respiration and metabolism (Lee et al. 1987; Li et al. 1988). Although some measurements of resting $[Ca^{2+}]_i$ and Ca^{2+} transient in cardiac myocytes have been made at room temperature (Stern et al. 1988; Takamatsu and Wier 1990), the effect of low temperature on $[Ca^{2+}]_i$ has not been investigated systematically.

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[1] A version of this chapter has been published: Liu, B., Wang, L. C. H. and Belke, D. D. (1991) Effect of low temperature on the cytosolic free Ca^{2+} in rat ventricular myocytes. *Cell Calcium,* 12, 11-18.

Low temperature has a profound effect on cardiac function. Ventricular fibrillation and heart failure are critical problems for non-hibernating species in hypothermia (Nordrehaug 1982; Johansson 1985, Solomon et al. 1989). Below 17°C, coronary flow and cardiac output are markedly decreased in isolated rat hearts (Burlington and Darvish 1988). Study of rat and rabbit ventricular papillary muscles shows aftercontractions and an increased resting tension at low temperature (Liu et al. 1990). A transient inward membrane current has also been observed in rat ventricular papillary muscles below 20°C (Liu et al. 1991). These events seem to arise from the spontaneous release of Ca^{2+} by the SR when the $[Ca^{2+}]_i$ becomes elevated, i.e. Ca^{2+} overload takes place in cardiac cells at low temperature. This rise in $[Ca^{2+}]_i$ may be one of the causes of severe cardiac arrhythmias in deep hypothermia.

To evaluate if temperature-dependent changes in $[Ca^{2+}]_i$ can indeed be correlated with cardiac contractile behaviour in non-hibernating species, we have used the fluorescent Ca^{2+} indicator, Indo-1, to measure the $[Ca^{2+}]_i$ in isolated rat ventricular myocytes at different temperatures. Since the compartmentalization of this fluorescent Ca^{2+} probe in mitochondria interferes with the accurate measurement of $[Ca^{2+}]_i$ (Williford et al. 1990; Spurgeon et al. 1990), the distribution of Indo-1 in cytosol and mitochondria has also been examined under our experimental conditions.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (200-250 g) were maintained individually with food and water ad libitum at 22°C and a 12:12 h light-dark photoperiod. All experimental procedures received prior approval by the University of Alberta Animal Use Committee and followed the guidelines of the Canadian Council on Animal Care.

Isolation of ventricular myocytes. Ventricular myocytes were isolated according to Ravens et al. (1989), with some modifications. Rats were anaesthetized intraperitoneally with sodium pentobarbital (80 mg/kg). The hearts were rapidly excised and immersed in cold (4°C) Ca^{2+}-free solution which contained (in mM): 100 NaCl, 10 KCl, 5.0 $MgSO_4$, 1.2 KH_2PO_4, 20 glucose, 50 taurine and 10 3-[N-Morpholino]-propanesulfonic acid (MOPS). The solution was previously aerated with $95\%O_2$-$5\%CO_2$ and the pH adjusted to 7.20. Following cannulation of the aorta, the hearts were perfused with Ca^{2+}-free solution at 37°C for 5 minutes at a rate of 10 ml/min with a peristaltic pump (Gilson miniplus II). This was followed by 12-15 minutes of perfusion with a collagenase solution comprised of 1% bovine serum albumin (BSA) (Boehringer Mannheim, Fraction V) and 1.0 mg/ml collagenase (Worthington, Type I, 151 unit/mg) in Ca^{2+}-free solution. The BSA was previously dialysed against 10 volumes of Ca^{2+}-free solution for 16 hours at 4°C. Following collagenase digestion, the ventricular tissue was incubated with fresh collagenase solution under gentle agitation at 37°C. The myocytes were harvested every 10 minutes by decanting the solution from remaining ventricular tissue. The solution was filtered through 6 layers of mesh gauze and centrifuged at room temperature for 1 minute at 20 x g_{max}. The myocytes were then washed once, purified 4 times in Ca^{2+}-free solution with 1% BSA by gravitative sedimentation, and diluted to about $3x10^5$ cells/ml at 37°C. About 90% of the myocytes in suspension showed a clear rod shape after [Ca^{2+}] in the suspension solution was gradually increased to 0.2 mM in order to further purify the myocytes after loading fluorescent Indo-1 into myocytes.

Loading of Indo-1 into myocytes. The acetoxymethyl esters of Indo-1 (Indo-1-AM, Molecular Probe, Eugene, Oregon) were dissolved in dimethylsulfoxide (DMSO) to a 1.0 mM concentration and stored at -80°C. To reduce cellular compartmentalization of Indo-1, a mixture of 77.5 μl fetal calf serum and 2.5 μl 25% Pluronic F-127 was added to 2.5 ml suspended myocytes solution 5 minutes before addition of 2.5 μl 1.0 mM Indo-1-AM. The loading of Indo-1 proceeded for 10 minutes in the dark at 37°C. The myocytes were then purified three times in the dark with a modified solution containing (in mM): 135 NaCl, 6.0 KCl, 1.0 $MgCl_2$, 10 MOPS, 10 taurine, 10 glucose, 0.2 $CaCl_2$ and 0.5% BSA, pH adjusted to 7.4 at 37°C.

At 37°C, the Ca^{2+} in the myocytes suspension solution was gradually increased from 0.2 to 1.0 mM before the measurement of Indo-1 fluorescence. For measurements at lower temperatures, the myocyte-suspension solution was first decreased to 15 or 5°C over a 20-30 minute period before raising the $[Ca^{2+}]$ to 1.0 mM. The myocytes were discarded if the viability was less than 80% in 1.0 mM Ca^{2+} solution at any temperature.

Measurement of $[Ca^{2+}]_i$. Dual emission fluorescence of Indo-1 was measured with an SLM AMINCO-8000C photon counting spectrofluorometer (SLM Instruments, Urbana, Illinois) outfitted with a T-optic system, and an IBM PS-2/50 computer. A bandpass of 4 nm was used for both excitation and emission monochrometers. The excitation wavelength was set at 350 nm. The first emission wavelength was selected using a 470 nm long pass filter (Schott KV-470) and the second emission wavelength was set at 400 nm by a monochrometer.

The myocytes suspended in 2.5 ml of solution were transferred into a temperature-controlled cuvette equipped with a magnetic stirrer. The changes of fluorescence at 400 nm (F_{400}) and 470 nm (F_{470}) were continuously monitored with the IBM computer. Seventy-five µl of $1x10^{-3}$ M digitonin (Sigma) was added to disrupt the sarcolemmal membrane and 70 µl of 3% Triton X-100 (BDH) was then added to lyse all subcellular membranes. The changes of fluorescence between measurements were due to Indo-1 present in the cytosol and all cellular compartments, respectively, when saturated by the prevailing 1.0 mM Ca^{2+}. To obtain the Ca^{2+}-free Indo-1 fluorescence, a 30 µl aliquot of 0.5 M ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) was then added to sequester the Ca^{2+}. The leakage of Indo-1 from the myocytes was determined by addition of EGTA in myocyte-suspension solution. The background signal of the solution (F_{back}) was measured with the suspension solution alone. The autofluorescence (F_{auto}) of the myocytes was detected with a suspension of Indo-1 unloaded myocytes. Both F_{back} and F_{auto} could be subtracted from the total fluorescence. The $[Ca^{2+}]_i$ was calculated with the equation (Grynkiewicz et al. 1985):

$$[Ca^{2+}]_i = K_d \frac{(R-R_{min})}{(R_{max}-R)} \beta \qquad (1)$$

where K_d is the effective dissociation constant, R is the ratio of F_{400} over F_{470}, R_{min} and R_{max} are F_{400}/F_{470} in zero Ca^{2+} and in saturating Ca^{2+}, respectively. ß is the ratio of F_{470} in zero Ca^{2+} over that in saturating Ca^{2+}.

To verify the effectiveness of digitonin and Triton X-100 in releasing Indo-1 from cytosol and mitochondria, the activities of marker enzymes representing the cytosolic (lactic dehydrogenase, LDH; Vassault 1983) and mitochondrial (citrate synthase, CS; Stritt 1984) compartments were measured in parallel experiments. After treatment with digitonin, the cell suspension was centrifuged and the resultant supernatant was assayed for LDH and CS activity. The pellet was resuspended, treated with Triton X-100, and subsequently assayed for LDH and CS. An aliquot of whole cells, prior to any treatment, was lysed with Triton X-100 to obtain total measurements of LDH and CS activities.

The Indo-1 fluorescence in Ca^{2+} solution at 37, 15 and 5°C was calibrated *in vitro* with 1.5 µM Indo-1 pentapotassium (Indo-1-K^+) (Molecular Probe, Eugene, Oregon) in a Ca^{2+}-EGTA buffer system, which contains (in mM): 115 KCl, 20 NaCl, 3.0 $MgCl_2$, 10 MOPS, 2.0 EGTA and various Ca^{2+}, pH adjusted to 7.00±0.01 at all temperatures with 30% KOH. A computer program (Fabiato and Fabiato 1979) was used to calculate the free Ca^{2+} concentration. The Ca^{2+}

association constants of EGTA were 2.978×10^6, 2.004×10^6 and 1.640×10^6 at 37, 15 and 5°C, respectively, in 0.15 M ionic strength (Harrison and Bers 1989). As the binding of fluorescent Ca^{2+} probe to cell membrane may change its Ca^{2+} reporting properties (Konishi et al. 1988), *in situ* calibration was performed with addition of membrane fractions of the myocytes, which had been pre-treated with Triton X-100 and washed in Ca^{2+}-EGTA solution before loading with 100 nM Indo-1-K^+.

Statistics. The data were presented as means ± SE. Multiple analysis was performed with a statistical program, SPSS/PC+(ANOVA), to compare the $[Ca^{2+}]_i$ values at 37, 15 and 5°C. P values < 0.05 were considered significant.

RESULTS

Calculation of dissociation constant (K_d) of Indo-1 at different temperatures and in situ calibration. An *in vitro* calibration with 1.5 μM Indo-1-K^+ showed that the maximal Indo-1 fluorescence in Ca^{2+}-free solution was at 474 nm, at all temperatures tested from 37 to 5°C. The maximal fluorescent value shifted to 398 nm when Indo-1 was saturated in 1.0 mM Ca^{2+}. At low temperatures the Indo-1 fluorescence curve in varying Ca^{2+} concentration shifted to the right (Fig.II.1) and the K_d value of Indo-1 to Ca^{2+} was increased from 368 nM at 37°C to 538 nM and 927 nM at 15 and 5°C, respectively. The changes of the Indo-1 fluorescence spectra in *in vitro* (Fig.II.2A) and *in situ* (Fig.II.2B) calibrations were compared. In the presence of membrane fractions of rat myocytes (Fig.II.2B), the emission wavelength for the maximal Indo-1 fluorescence shifted from 474 nm to 465 nm in Ca^{2+}-free solution, but did not change in 1.0 mM Ca^{2+} solution. The K_d value increased 20% over that measured in the absence of myocyte fractions. Therefore, all subsequent calculations presented herein were based on the *in situ* calibration curves.

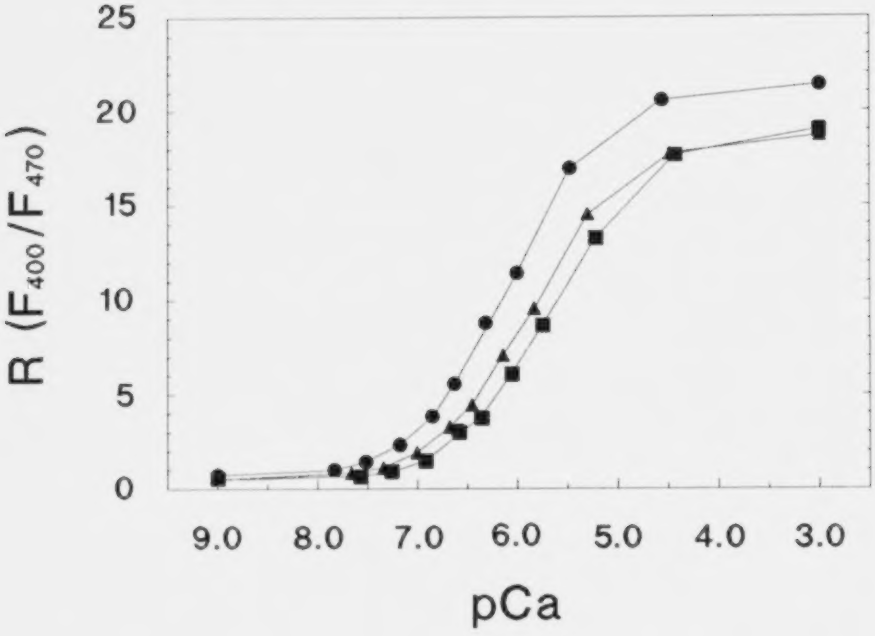

Figure II.1. Ratio measurement of 1.5 μM Indo-1 pentapotassium (Indo-1-K$^+$) in different free [Ca^{2+}] at 37°C (filled circles), 15°C (filled triangles) and 5°C (filled squares). The ordinate is the ratio (R) of fluorescence at 400 nm over that at 470 nm (F_{400}/F_{470}). The abscissa is Ca^{2+} concentration in -log value (pCa). The K_d is 368 nM at 37°C, 538 nM at 15°C, and 927 nM at 5°C, respectively.

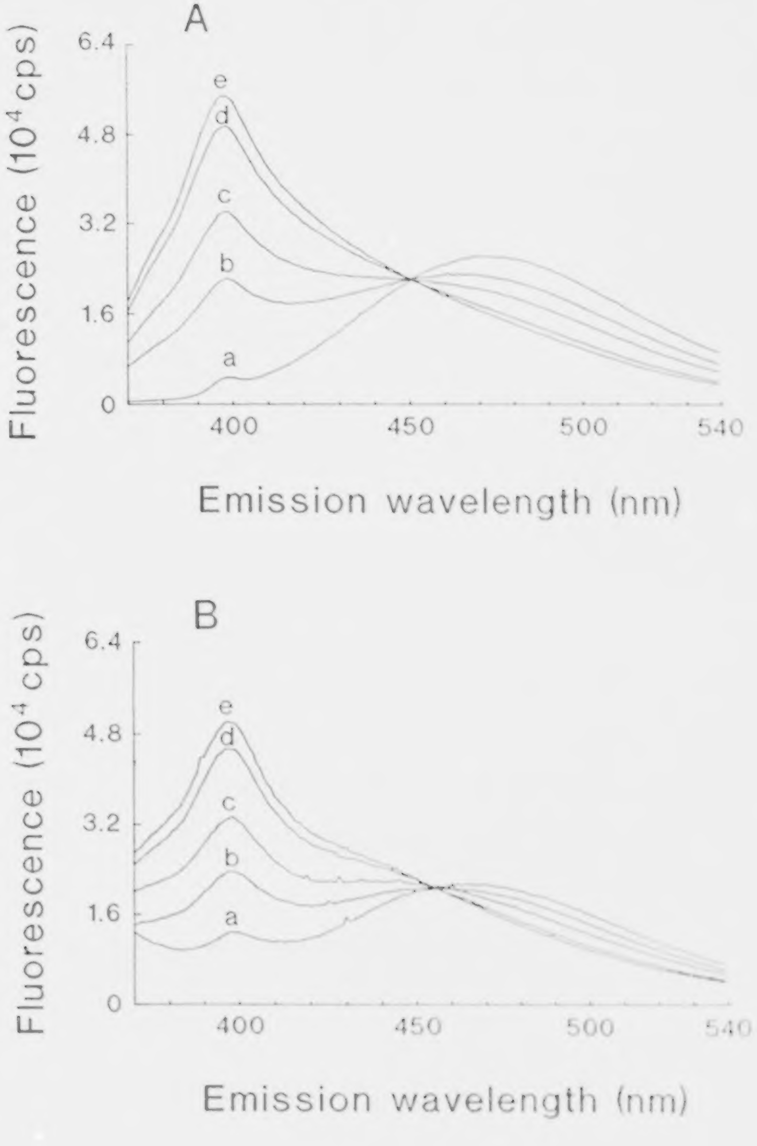

Figure II.2. Emission spectra for 100 nM Indo-1-K$^+$ as a function of free Ca^{2+} at 37°C in the absence (A) and presence (B) of membrane fractions of the myocytes. pH was set at 7.00±0.01 with KOH. Excitation was 350 nm. The abscissa is the emission wavelength. The ordinate is fluorescence photon counts per second (cps). The free Ca^{2+} concentration: (a) 0 nM, (b) 250 nM, (c) 1.05 μM, (d) 25.14 μM, (e) 1.00 mM.

Leakage of the fluorescent Indo-1 from myocytes into suspension solution. Leakage was detected by addition of 2 mM EGTA to the myocyte suspension solution. When the extracellular Ca^{2+} was sequestered by EGTA, the resulting initial rapid change in fluorescence (<3 seconds) indicated the presence of extracellular dye. As shown in Figure II.3, the F_{400} increased from 1.598×10^4 to 1.689×10^4 counts/s and F_{470} decreased from 1.511×10^4 to 1.450×10^4 counts/s when fluorescence was measured at 37°C immediately after washing the myocytes and at a time point 15 minutes later. These time-dependent changes of F_{400} and F_{470} amounted to 5.7 and 4.1%, respectively, from their initial values. The total change in the ratio of F_{400} over F_{470} was less than 10% at 37°C. At lower temperatures, 15 and 5°C, this leakage was less than that at 37°C. Accordingly, all our measurements of $[Ca^{2+}]_i$ were done within 15 minutes after washing at all temperatures.

$[Ca^{2+}]_i$ in ventricular myocytes. A typical recording of the $[Ca^{2+}]_i$ measured at 37°C is shown in Figure II.4. The total resting F_{400} was 2.269×10^4 counts/s in which F_{back} was 4.818×10^3 counts/s and F_{auto} was 1.356×10^4 counts/s. The total resting F_{470} was 2.067×10^4 counts/s in which F_{back} was 2.364×10^3 counts/s and F_{auto} was 1.083×10^4 counts/s. When the $[K^+]$ in cuvette was increased to 60 mM, the F_{400} increased and the F_{470} decreased. As the Triton X-100 was added to disrupt all cell membranes, the F_{400} increased to the maximum and the F_{470} decreased and approached the baseline at the same time. When EGTA was added to sequester the Ca^{2+}, the F_{400} decreased to the baseline and the F_{470} increased to the maximum. The mean value of total cellular $[Ca^{2+}]_i$ measured in resting state was 134 ± 8 nM (n=10; range 99-190 nM) at 37°C, 246 ± 22 nM (n=10; range 177-397 nM) at 15°C and 285 ± 17 nM (n=8; range 214-362 nM) at 5°C, respectively.

The changes in Indo-1 fluorescence at 37°C after treatment with digitonin and Triton X-100 are shown in Figure II.5. F_{400} increased and F_{470} decreased markedly when 30 μM digitonin was added. Further increase in F_{400} and decrease

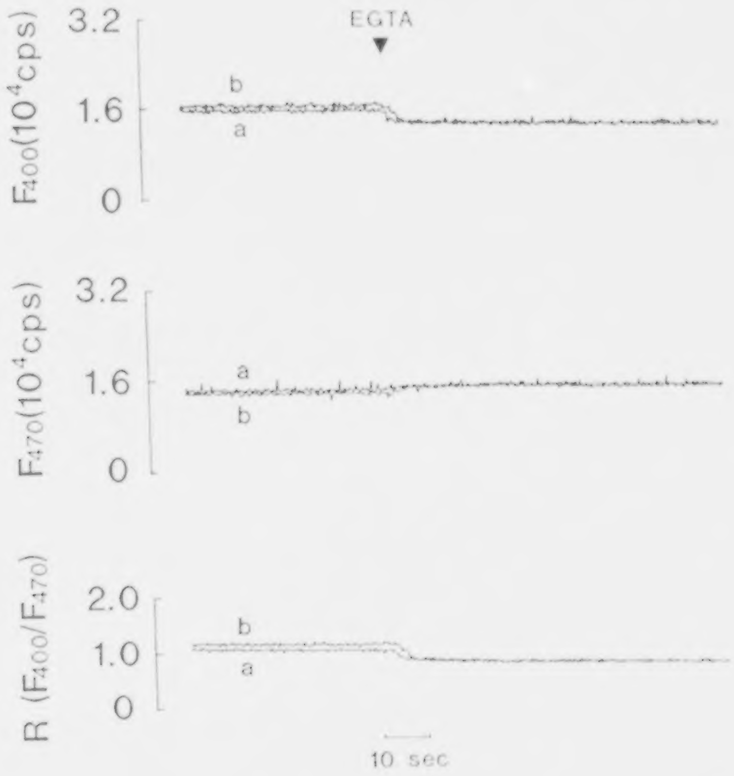

Figure II.3. Time course of fluorescent Indo-1 leakage from cells at 37°C: (a) measured immediately after wash, (b) 15 minutes later.

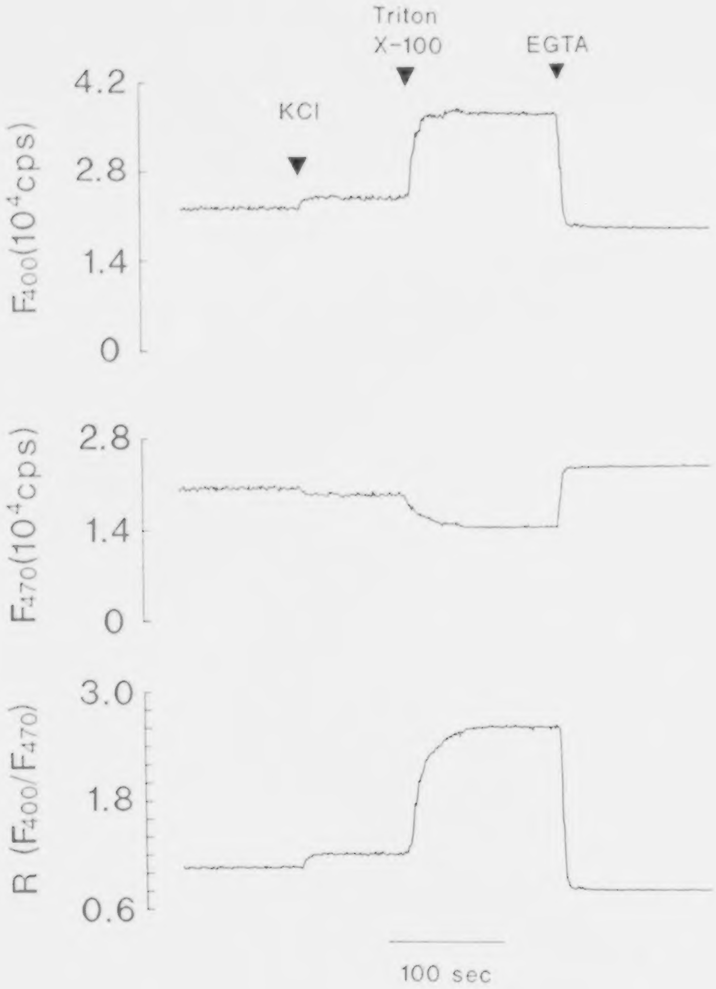

Figure II.4. A representative recording of Indo-1 fluorescence before and after additions of 75 µl 2.0 M KCl, 70 µl 3% Triton X-100 and 30 µl 0.5 M EGTA in 2.5 ml suspended myocytes at 37°C.

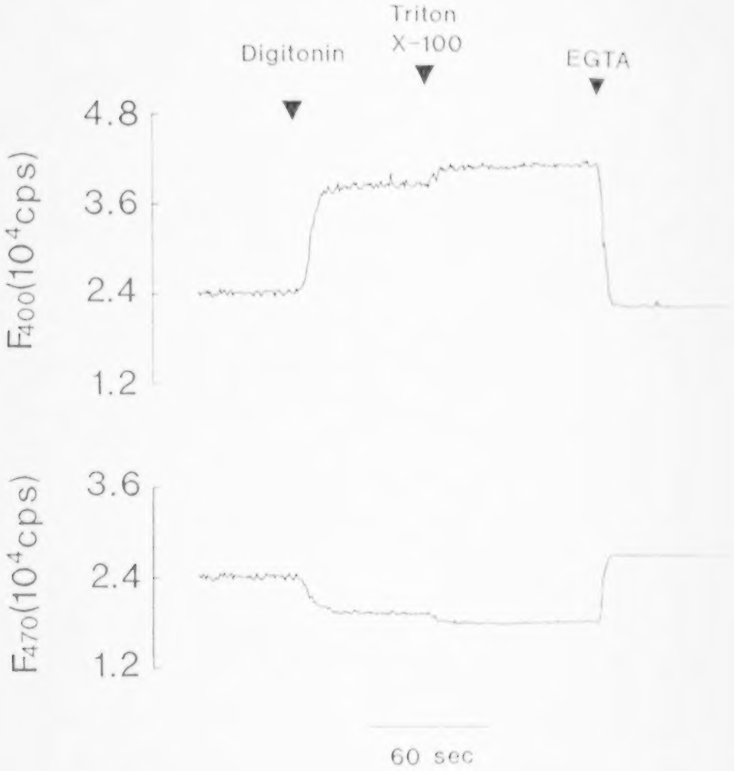

Figure II.5. A representative recording on changes of Indo-1 fluorescence after additions of 75 μl 1.0 mM digitonin, 70 μl 3% Triton X-100 and 30 μl 0.5 M EGTA in 2.5 ml suspended myocytes at 37°C.

in F_{470} was observed when Triton X-100 was added. The distributions of Indo-1 in cytosol (X) and in mitochondria (Y) were therefore calculated according to the following equations:

$$a_1 \cdot X + b_1 \cdot Y = \text{delta } F_{digi} \qquad (2)$$

$$a_2 \cdot X + b_2 \cdot Y = \text{delta } F_{triton} \qquad (3)$$

where a_1 and b_1 are the percentages of Indo-1 released by 30 μM digitonin from cytosol and mitochondria, respectively. a_2 and b_2 are the percentages of remaining Indo-1 released from cytosol and mitochondria after the addition of Triton X-100. Delta F_{digi} and delta F_{triton} are the changes of fluorescence due to 30 μM digitonin and Triton X-100, respectively. As 97.7±0.3% (n=7) of cytosolic LDH and 7.7±0.3% (n=6) of mitochondrial CS were released by 30 μM digitonin, we assumed that similar proportions of Indo-1 were released from the two compartments. The distribution of Indo-1 between the two compartments was 89±2% in cytosol and 11±2% in mitochondria (seven similar measurements at 37°C). Cooling the cells to 15 and 5°C did not influence the distribution of Indo-1 between the two compartments (five measurements at each temperature).

Assuming the mitochondrial $[Ca^{2+}]_i$ to be 66% of the cytosolic value when cytosolic $[Ca^{2+}]_i$ is less than 1 μM (Spurgeon et al. 1990), we calculated the cytosolic $[Ca^{2+}]_i$ using the following equation:

$$c_1 \cdot X + c_2 \cdot Y = [Ca^{2+}]_{i\text{-}total} \qquad (4)$$

where X and Y are cytosolic and mitochondrial $[Ca^{2+}]_i$, respectively, and c_1 and c_2 are distributions of Indo-1 in cytosol and mitochondria, respectively. By subtracting mitochondrial $[Ca^{2+}]_i$ from the total $[Ca^{2+}]_i$, we estimated that the cytosolic $[Ca^{2+}]_i$ in the resting state was 139±8 nM (n=10; range 103-198 nM) at 37°C (Fig.II.6). When temperature was decreased to 15 and 5°C, the $[Ca^{2+}]_i$ increased significantly ($p<0.05$) to 255±23 nM (n=10; range 183-413 nM) and 297±18 nM (n=8; range 222-376 nM), respectively.

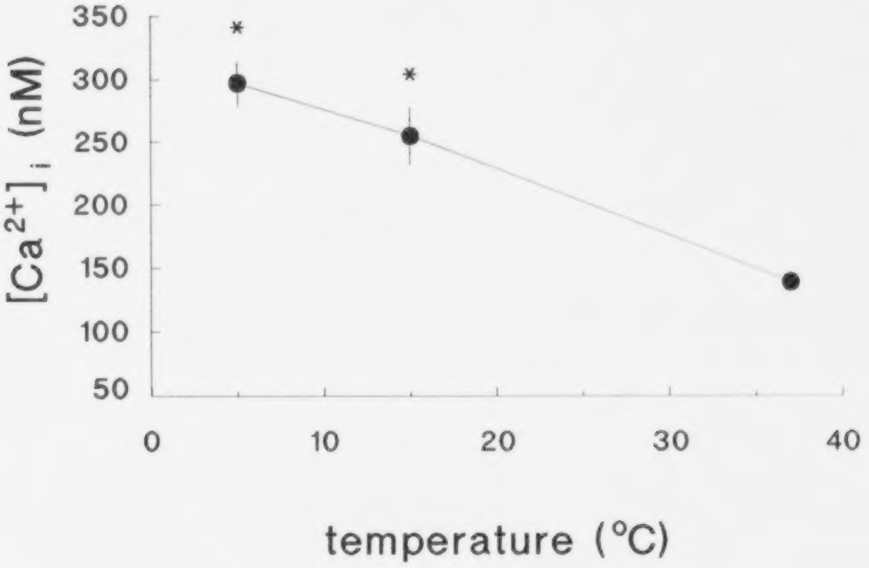

Figure II.6. Changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) with temperature in isolated rat ventricular myocytes. means±SE, n=8-10.

DISCUSSION

Loading of Indo-1 into the cells is probably the most critical procedure in the experiments. Because the free acid of Indo-1 is a Ca^{2+} chelator, it can act as a Ca^{2+} buffer in the cell and overloading can lead to altered cell functions (duBell et al. 1988). In our pilot experiments, the myocytes were incubated with 3 μM Indo-1-AM in 1.0 mM Ca^{2+} for 20 minutes at 37°C. Only about 60% of the myocytes had a clear rod shape after the incubation. This was probably due to overloading with Indo-1 which buffered too much Ca^{2+} in the cell and caused cell death. Further experiments indicated that loading in 0.2 mM Ca^{2+} at 37°C for 10 minutes and using less than 1 μM Indo-1-AM in the incubation resulted in greater than 75% cell survival and a satisfactory Indo-1 fluorescence signal.

Cellular compartmentalization of Indo-1 is known to interfere with cytosolic $[Ca^{2+}]_i$ measurement. In a recent study, 51% of the Indo-1 was found in mitochondria when the rat myocytes were incubated with Indo-1-AM (Spurgeon et al. 1990). Addition of Pluronic F-127, which is a non-ionic dispersing agent that helps solubilize large dye molecules in physiological media (Poenie et al. 1986), reduced the amount of Indo-1 in mitochondria to 11% in the present study. An assumption used for calculation in the present study is that the mitochondrial $[Ca^{2+}]_i$ is 66% of the cytosolic value when cytosolic $[Ca^{2+}]_i$ is less than 1 μM. This assumption is based upon an unpublished study, using isolated mitochondria, cited in the report of Spurgeon et al. (1990). If this relationship between cytosolic and mitochondrial $[Ca^{2+}]_i$ should shift by 50%, our calculated cytosolic $[Ca^{2+}]_i$ value using equation (4) will differ less than 5%. A recent study on single intact myocytes shows that mitochondrial and cytosolic $[Ca^{2+}]_i$ values are 83 nM and 149 nM, respectively, in rat ventricular myocytes measured with fluorescent Indo-1 at 23°C. The ratio of mitochondrial vs. cytosolic $[Ca^{2+}]_i$ is always less than unity unless cytosolic $[Ca^{2+}]_i$ exceeds 500 nM (Miyata et al. 1991).

Unlike fluorescence measurement of cytosolic $[Ca^{2+}]_i$ in single cells, the interference due to leakage of loaded Indo-1 from a population of myocytes cannot be avoided because of cell injury during isolation. A dual sample protocol with addition of Mn^{2+} has been proposed to quench extracellular Fura-2 leaked from cells during the measurement of $[Ca^{2+}]_i$ (McDonough and Button 1989). However, the corrected value of $[Ca^{2+}]_i$ might be an underestimation because the added Mn^{2+} can enter into cells and quench the fluorescence of intracellular Fura-2. In our experiments, we used EGTA instead to detect any leaked Indo-1 (Fig.II.4). As shown in Figures II.4 and II.5, the resting fluorescence in our experiments was quite stable at 37°C, indicating that the leakage of Indo-1 was less than 10% within the 15 minute measurement time. The $[Ca^{2+}]_i$ of rat ventricular myocytes measured in suspension at 37°C in the present study (139±8 nM) is comparable with an early report (134±43 nM) measured with Fura-2 in single cells at 35°C (Wier et al. 1987).

The maintenance of cardiac $[Ca^{2+}]_i$ at a low level in the resting state involves Ca^{2+} uptake by SR and mitochondria, and Ca^{2+} extrusion from cell via the sarcolemmal Na^+-Ca^{2+} exchanger and Ca^{2+}-ATPase. Evidence has indicated that during cardiac relaxation, reduction of $[Ca^{2+}]_i$ by SR is 3-4 times faster than the Na^+-Ca^{2+} exchange, which is in turn 35-50 times faster than either mitochondrial Ca^{2+} uptake or Ca^{2+} extrusion by the Ca^{2+}-ATPase out of the sarcolemma. This suggests a primary role of SR Ca^{2+} uptake and sarcolemmal Na^+-Ca^{2+} exchange in the regulation of $[Ca^{2+}]_i$ (Bers et al. 1992). Recent studies have shown a significant increase of intracellular Na^+ at low temperature in ferret papillary muscles (Chapman 1986) and in chick embryonic heart cells (Navas et al. 1990), due to inhibitory effect of low temperature on the Na^+-K^+-ATPase in sarcolemmal membrane (Thurston et al. 1978). This suggests that Ca^{2+} extrusion via the Na^+-Ca^{2+} exchange will be inhibited markedly while Ca^{2+} influx via the Na^+-Ca^{2+} exchange will be promoted at low temperature due to an increase of intracellular Na^+ in combination with a marked depolarization of membrane in non-hibernating species (Webb et al. 1969; Chapman 1986). This will lead to a significant increase in $[Ca^{2+}]_i$, as seen in the present study, and in total Ca^{2+} content (Navas et al. 1990) at low temperatures. Under these conditions, the SR would be overloaded with Ca^{2+} and spontaneous contractions could occur if spontaneous Ca^{2+}-induced Ca^{2+} release occurs (Lakatta 1992). Indeed, we observed frequent spontaneous contractions in some rat myocytes at 15°C. At 5°C almost every rat myocyte developed frequent spontaneous contractions when examined under the microscope. Under these circumstances, what we measured at low temperature, particularly at 5°C, is an instantaneous $[Ca^{2+}]_i$ rather than the value in resting state because the $[Ca^{2+}]_i$ undergoes asynchronous spatio-temporal fluctuations (Kort et al. 1985). Since spontaneous release of Ca^{2+} from the SR is the major source for the fluctuations (Lakatta et al. 1985), further studies on the effect of ryanodine on $[Ca^{2+}]_i$ at low temperature may provide further information about the $[Ca^{2+}]_i$ in resting state and the contribution of SR Ca^{2+} to cardiac arrhythmia at low temperature.

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III. REGULATION OF CARDIAC CYTOSOLIC FREE Ca^{2+} AND MUSCLE CONTRACTION AT LOW TEMPERATURE IN THE RICHARDSON'S GROUND SQUIRREL [2]

INTRODUCTION

Previous studies have indicated that cardiac arrhythmias, particularly ventricular fibrillation, appear in most nonhibernating species if body temperature is decreased to between 30 and 15°C (Nordrehaug 1982; Solomon et al. 1989). In isolated ventricular muscles from rats and rabbits, aftercontractions and an increase in resting tension are observed when the muscle is cooled from 37°C to about 15°C; both effects indicate that a disturbance in regulation of intracellular Ca^{2+} is responsible for cardiac arrhythmia and/or dysfunction in the cold (Liu et al. 1990). Direct measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) with the fluorescent dye Indo-1 has shown that $[Ca^{2+}]_i$ in isolated rat ventricular myocytes increases significantly when temperature is decreased from 37°C to 15 and 5°C (Liu et al. 1991b). In contrast, the hearts of mammalian hibernators rarely develop severe cardiac arrhythmias upon cooling, and competent cardiac contraction can be maintained near 0°C at a rate of 2-10 beats/min (Lyman and Blinks 1959; Caprette and Senturia 1984; Burlington et al. 1989). Since $[Ca^{2+}]_i$ plays a central role in cardiac excitation-contraction coupling, our present objective is to document the changes in cardiac $[Ca^{2+}]_i$ in a mammalian hibernating species (Richardson's ground squirrel) at low temperatures. Since the Na^+-Ca^{2+} exchange mechanism contributes significantly to $[Ca^{2+}]_i$ regulation (Noble 1984; Kimura et al. 1986; Ravens and Wettwer

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[2] A version of this chapter has been accepted for publication: Liu, B. and Wang, L. C. H. (1993) Regulation of cardiac cytosolic free Ca^{2+} at low temperature in the Richardson's ground squirrel. In: *Life in the Cold III: Ecological, Physiological, and Molecular Mechanisms.* eds. Carey, C., Florant, G. L., Wunder, B. A. and Horwitz, B. Westview Press.

1989), its status at low temperature has also been investigated. This has been done at 5°C by reducing extracellular Na^+ ($[Na^+]_o$). This reduces the Ca^{2+} extrusion via Na^+-Ca^{2+} exchange since the activity of the Na^+-Ca^{2+} exchanger is highly dependent on the Na^+ gradient across the sarcolemmal membrane. The contractile changes in response to decreasing $[Na^+]_o$ have been studied in isolated papillary muscles and the changes in $[Ca^{2+}]_i$ measured in isolated ventricular myocytes with fluorescent Indo-1.

MATERIALS AND METHODS

All experimental procedures reported herein received prior approval by the University of Alberta Animal Use Committee following the guidelines of the Canadian Council on Animal Care.

Animals. Richardson's ground squirrels (*Spermophilus richardsonii*) were trapped in farm fields near Edmonton, Alberta, Canada. They were maintained individually with food and water ad libitum at 22°C and 12:12 h light-dark photoperiod.

Isolation of ventricular myocytes. Active individuals (350-600 g) with rectal temperature around 37°C were sacrificed by decapitation. The procedures of isolating ventricular myocytes were similar to those in rats (Liu et al. 1991b) except that the initial perfusion at 37°C with Ca^{2+}-free solution was prolonged to 10-12 minutes rather than 5 minutes. About 70-80% of the myocytes in suspension showed a typical rod shape after $[Ca^{2+}]$ in the suspension solution was gradually increased to 0.2 mM.

Measurement of $[Ca^{2+}]_i$ using fluorescent Indo-1. The procedures for loading of Indo-1 into cells, determination of cellular Indo-1 compartmentalization, *in situ* calibration of Indo-1 dissociation constants, and the measurement of $[Ca^{2+}]_i$ have been described in detail in a previous study of rats (Liu et al. 1991b) and in Chapter II.

Isometric contraction of isolated papillary muscles at 5°C. As soon as the hearts were excised, they were rapidly immersed in cold (4°C) Krebs-Henseleit (K-H) solution which contained (in mM): 118 NaCl, 4.7 KCl, 1.6 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.0 $CaCl_2$ and 11 glucose, and which was aerated with 95%O_2 and 5%CO_2 (pH 7.4). Right ventricular papillary muscles were excised under a dissecting microscope (Vickers) and mounted vertically in 5 ml thermostated bath chambers. The ventricular base was tied with 5-0 thread to the holder of an electrode and the tendinous end to a force transducer (Grass FT03C). The muscles were first equilibrated at 37°C for 60 minutes during which time the muscles were stimulated with a WPI A310 Accupulser and stretched until maximal contractions were reached. The stimulus pulse was 0.2 Hz; each pulse was 5 ms in duration and 10 mA in intensity. The bath temperature was then gradually decreased to 5°C over a 50 minute period. The muscles were continuously stimulated at 0.2 Hz at 5°C for another 60 minutes before the contraction signals were recorded with a Grass polygraph (model 7D). The data were stored in an IBM PC with CODAS, a data acquisition program (DATAQ Instruments Inc), at a sampling rate of 1 kHz for later analysis.

Cellular energy metabolism at 5°C was interrupted by an uncoupler of mitochondrial oxidative phosphorylation, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 20 μM, Sigma). Ca^{2+} extrusion via Na^+-Ca^{2+} exchange at 5°C was reduced by a decrease in $[Na^+]_o$ which was substituted isosmotically by either Li^+ (LiCl, Sigma) or sucrose (BDH).

Statistics. Results are presented as means ± SE. Multiple comparisons with a statistical program, SPSS/PC+(ANOVA) were made on $[Ca^{2+}]_i$ values at 37, 15 and 5°C, as well as on the contractile changes after 40%, 60%, 80% and 90% of $[Na^+]_o$ were substituted by either Li^+ or sucrose. The $[Ca^{2+}]_i$ values at 5°C were compared in normal K-H solution versus the 90% Na^+ substituted solution by student's *t* test. P values < 0.05 were considered significant.

RESULTS

$[Ca^{2+}]_i$ in ventricular myocytes. $[Ca^{2+}]_i$ was 103±4 nM (n=13) at 37°C and increased to 138±12 nM (n=10) and 168±16 nM (n=11), respectively, when temperature was decreased to 15 and 5°C (Fig.III.1). Although there was no significant difference in $[Ca^{2+}]_i$ between 37 and 15°C, and 15 and 5°C, the mean value of $[Ca^{2+}]_i$ at 5°C was significantly higher than that at 37°C.

$[Ca^{2+}]_i$ after addition of CCCP. When cellular energy metabolism was interrupted at 5°C, two phases in the changes of Indo-1 fluorescence and therefore $[Ca^{2+}]_i$ were observed. A typical recording of the changes of Indo-1 fluorescence at 400 nm (F_{400}) and 470 nm (F_{470}) is shown in Figure III.2. When 20 μM CCCP was added to myocytes suspension at 5°C, F_{400} increased rapidly within 30 seconds and then continued to increase gradually in the next 3-5 minutes; the opposite changes occurred in F_{470}. Calculations based on the equation of Grynkiewicz et al. (1985) indicated that $[Ca^{2+}]_i$ increased from 170 nM in normal K-H solution to 648 nM and 1.3 μM, respectively, corresponding to the two phasic changes of Indo-1 fluorescence after 20 μM CCCP at 5°C. The mean value (594±65 nM; n=8) for $[Ca^{2+}]_i$ at 5°C after treatment with 20 μM CCCP for 30 seconds was significantly ($p<0.05$) higher than the control value (168±16 nM; n=11) at 5°C. Addition of 30 μM digitonin destroyed sarcolemmal membrane and induced a significant Ca^{2+} influx from the medium (1.0 mM) so that there was a further change in Indo-1 fluorescence. Addition of Triton X-100 dissolved all membrane structures but produced no further changes in Indo-1 fluorescence, indicating that the fluorescent Indo-1 loaded using our particular procedure was mainly in the cytosol rather than being sequestered in organelles. Addition of EGTA in suspension chelated all Ca^{2+} so that Indo-1 fluorescence in zero Ca^{2+} was obtained.

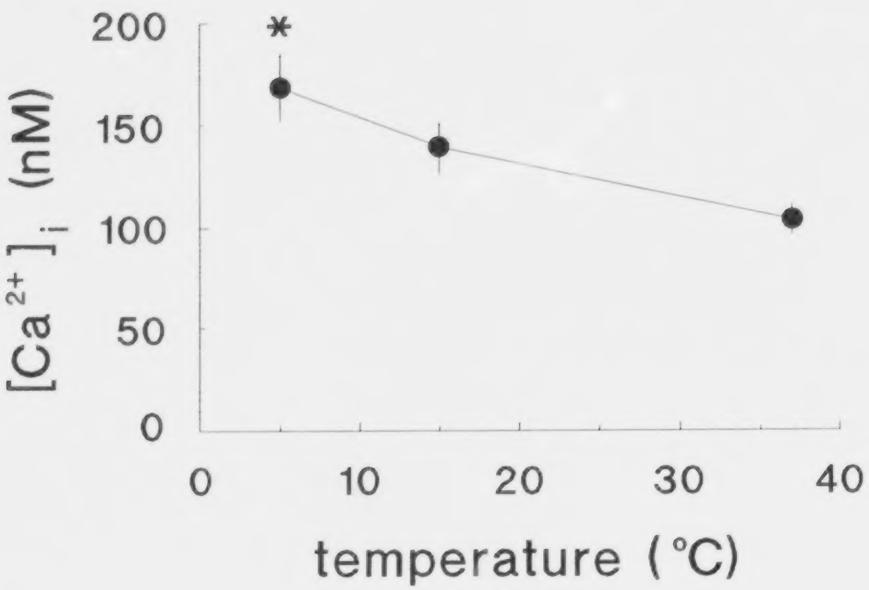

Figure III.1. Effect of temperature on cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) of isolated ventricular myocytes from active Richardson's ground squirrels. * significantly different from the value at 37°C. means±SE, n=10-13 at each temperature.

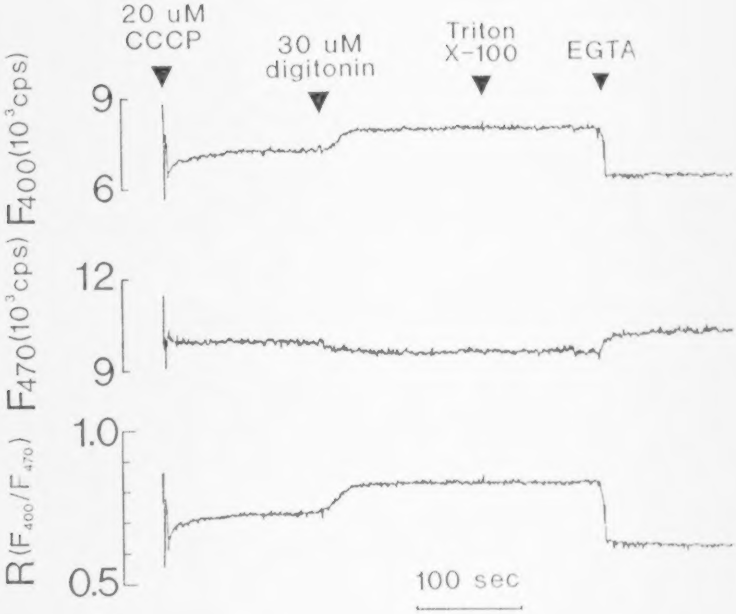

Figure III.2. Typical recordings for the measurement of $[Ca^{2+}]_i$ after addition of 20 μM CCCP at 5°C.

Effects of decreased $[Na^+]_o$ *on muscle contraction and* $[Ca^{2+}]_i$ *at 5°C.* When $[Na^+]_o$ was reduced by 20%, very little change in muscle contraction was observed at 5°C. However, when $[Na^+]_o$ was further decreased, both the resting tension and the magnitude of muscle contraction increased in a $[Na^+]_o$-dependent manner. A representative recording of contractile changes at 5°C is shown in Figure III.3. When 40%, 60% and 80% of $[Na^+]_o$ were substituted by Li^+ for 10 minutes, the resting tension increased by 10±1%, 18±1% and 31±%, respectively (Fig.III.4), and the increases in muscle contraction were 34±11%, 69±13% and 78±14%, respectively (Fig.III.5). With further substitution of $[Na^+]_o$ up to 90%, the resting tension increased even more markedly while the magnitude of the muscle contraction decreased to about 83±2% of the control value. The mean value of 541±32 nM (n=9) for $[Ca^{2+}]_i$ at 5°C after 90% substitution of $[Na^+]_o$ by Li^+ (Fig.III.6) was significantly ($p<0.05$) higher than the control (168±16 nM; n=11).

Similar changes in muscle contraction at 5°C were also observed when 40, 60, and 80% of $[Na^+]_o$ were substituted with sucrose (Fig.III.7). Arrhythmic aftercontractions were observed at 5°C when 80% of $[Na^+]_o$ was substituted by sucrose for over 30 minutes (Fig.III.8).

DISCUSSION

The effects of low $[Na^+]_o$ on cardiac muscle contraction and $[Ca^{2+}]_i$ have been studied in many non-hibernating species. An increase in both resting tension and amplitude of contraction is observed when $[Na^+]_o$ is substituted with either Li^+ or sucrose, suggesting an excessive increase in $[Ca^{2+}]_i$ (Chapman 1983; Sonn and Lee 1988). Indeed, both $[Ca^{2+}]_i$ (Sheu and Fozzard 1982; Allen et al. 1983) and total tissue Ca^{2+} content (Lüllmann et al. 1991) have been shown to increase markedly after these treatments. Since a decrease in $[Na^+]_o$ will reduce Ca^{2+} extrusion via the Na^+-Ca^{2+} exchanger, it has been proposed that a decrease in Na^+-Ca^{2+} exchanger activity is responsible for the observed increase of

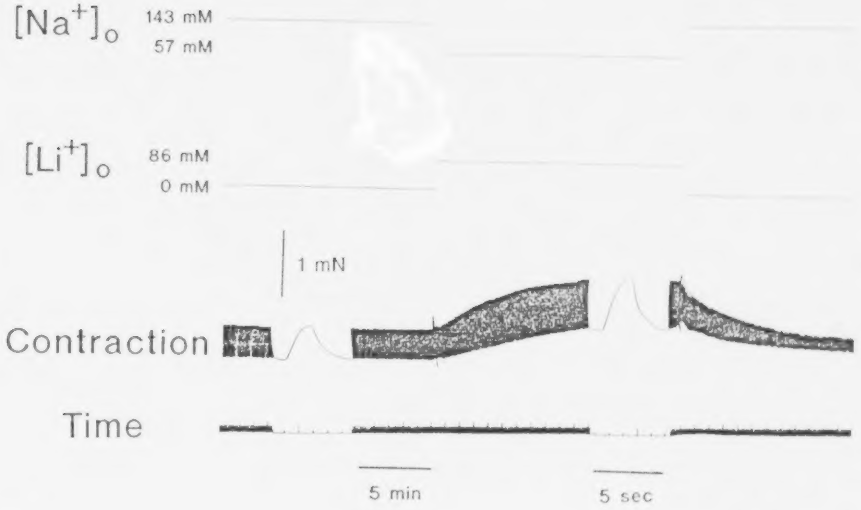

Figure III.3. A representative recording of cardiac contractile changes of ground squirrel at 5°C when 60% of extracellular Na^+ ($[Na^+]_o$) was substituted by Li^+ ($[Li^+]_o$) isosmotically.

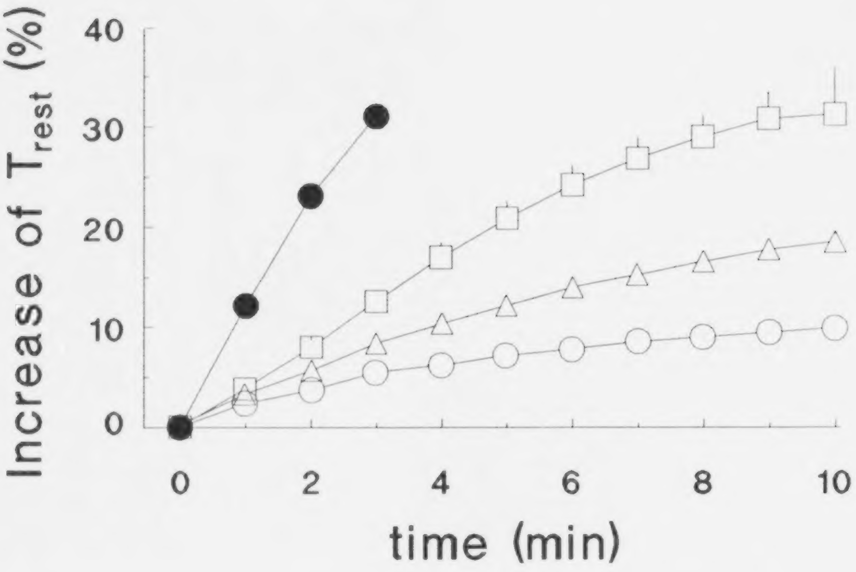

Figure III.4. Changes in resting tension (T_{rest}) of cardiac muscles of ground squirrels at 5°C when extracellular Na^+ was substituted with Li^+ by 40% (open circles), 60% (open triangles), 80% (open squares), and 90% (filled circles) for 10 minutes. means±SE, n=8-10.

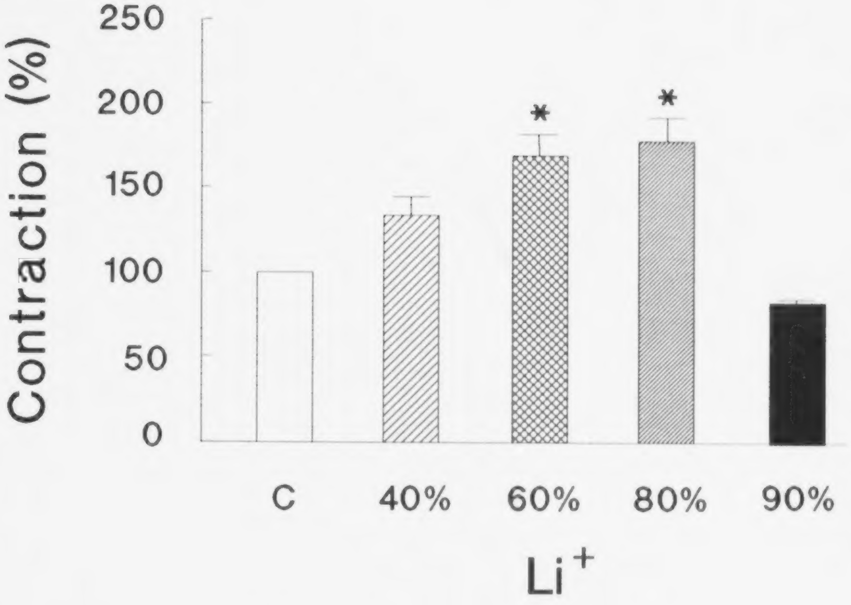

Figure III.5. Changes in cardiac muscle contractions of ground squirrels at 5°C before (C) and after extracellular Na^+ was substituted by Li^+ for 10 minutes. * significantly different ($p<0.05$) from the control. means+SE, n=7-9.

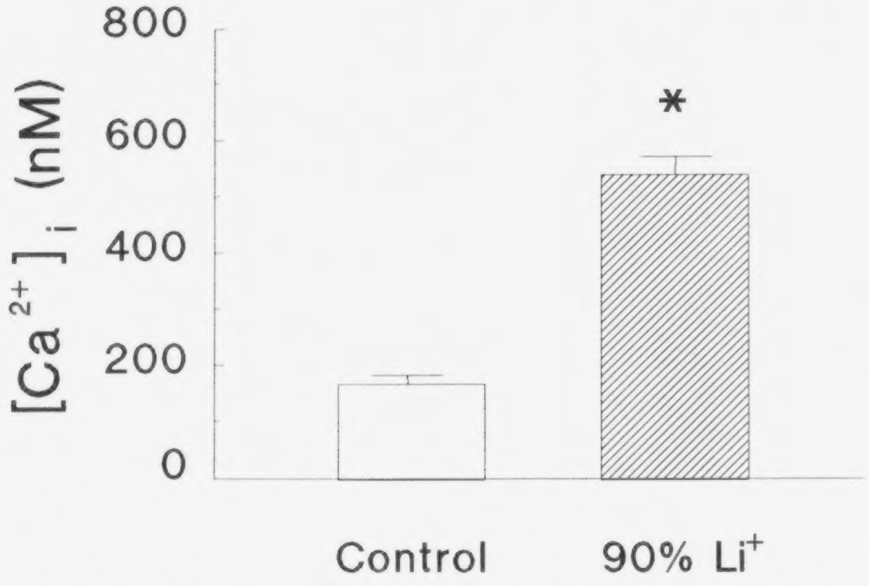

Figure III.6. Changes in cardiac cytosolic free Ca^{2+} of ground squirrels at 5°C after 90% of extracellular Na^+ was substituted by Li^+. * significantly different ($p<0.05$) from the control. means±SE, n=9-11.

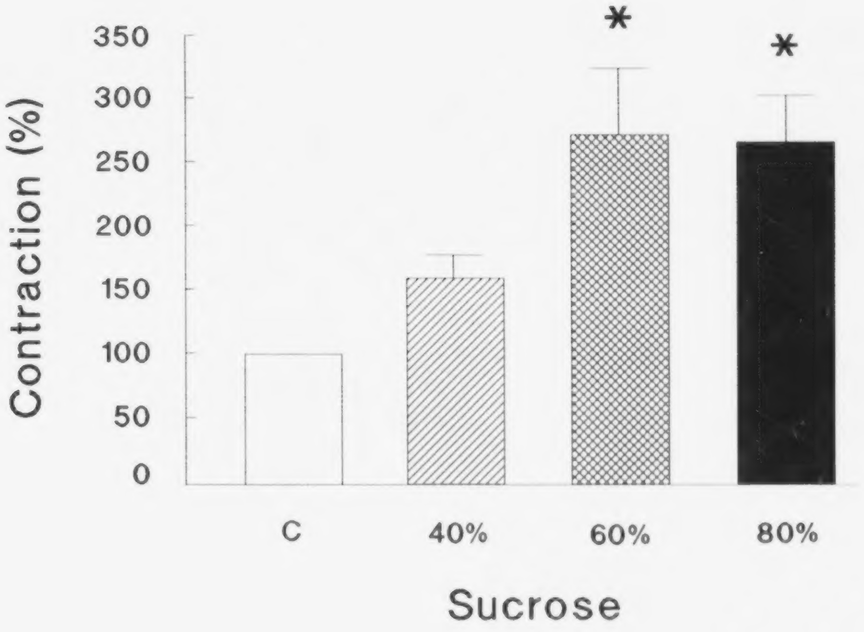

Figure III.7. Changes in cardiac muscle contractions of ground squirrels at 5°C before (C) and after extracellular Na^+ was substituted by sucrose for 10 minutes. * significantly different ($p<0.05$) from the control. means±SE, n=6-9.

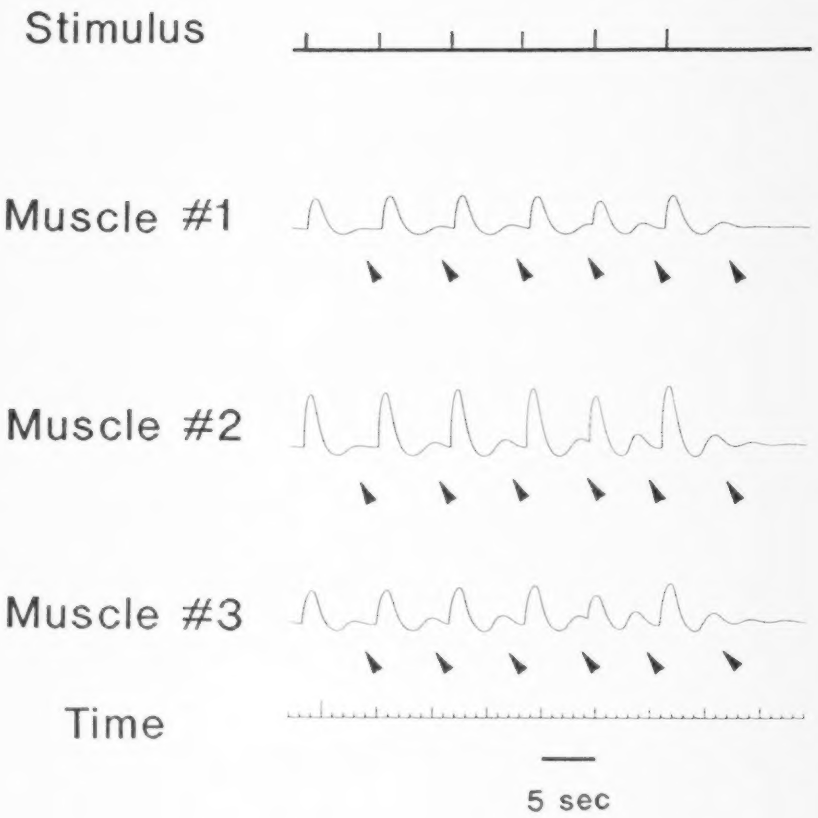

Figure III.8. Aftercontractions (as indicated by arrows) at 5°C in cardiac muscles of ground squirrels after 80% of extracellular Na^+ was substituted by sucrose for 30 minutes.

$[Ca^{2+}]_i$ upon lowering $[Na^+]_o$.

It has been well established that the precise regulation of $[Ca^{2+}]_i$ plays an important role in heart function (Katz and Reuter 1979). An abnormal increase in $[Ca^{2+}]_i$ activates energy dependent processes and cellular phospholipases, leading to energy depletion and membrane damage, respectively (Farber 1981, Tani 1990). Further, an excessive rise in $[Ca^{2+}]_i$ induces a transient inward current which triggers afterdepolarizations and aftercontractions leading to severe cardiac arrhythmias (Cranefield and Wit 1979). In a previous study from our laboratory, $[Ca^{2+}]_i$ in isolated ventricular myocytes from rats increased significantly from 139 nM at 37°C to about 300 nM at 5°C (Liu et al. 1991b). This marked increase in $[Ca^{2+}]_i$ may be responsible for the transient inward current (Liu et al. 1991a), and aftercontractions in isolated muscles occurring at low temperature in this species (Liu et al. 1990). In contrast, the cardiac $[Ca^{2+}]_i$ in Richardson's ground squirrels increases only 63% when temperature is decreased from 37 to 5°C (from 103 nM to 168 nM). The $[Ca^{2+}]_i$ at 5°C in ground squirrels is significantly lower than that in rats, indicating a superior ability to regulate $[Ca^{2+}]_i$ in hibernators as opposed to non-hibernators.

It is generally accepted that three mechanisms are involved in the regulation of $[Ca^{2+}]_i$: Ca^{2+} uptake by sarcoplasmic reticulum (SR), Ca^{2+} extrusion via Na^+-Ca^{2+} exchange and Ca^{2+} extrusion by sarcolemmal Ca^{2+}-ATPase. Evidence has indicated that the rate of Ca^{2+} uptake by cardiac SR in hibernators is significantly higher in the hibernating state than in the active state (Belke et al. 1987; Belke et al. 1991). This has been referred to as a specific seasonal adaptation commensurate with hibernation (Wang 1988). In the present study, the role of Na^+-Ca^{2+} exchange in regulating $[Ca^{2+}]_i$ at low temperature has been evaluated by reduction of $[Na^+]_o$. When $[Na^+]_o$ is substituted with up to 90% Li^+ to reduce Ca^{2+} extrusion via the Na^+-Ca^{2+} exchange at 5°C, the resting tension of ventricular muscles as well as the $[Ca^{2+}]_i$ increases markedly. The observed reduction in muscle contraction as $[Na^+]_o$ is substituted with Li^+ to 90% is consistent with a previous study which shows a decline in tension development in chemically skinned cardiac muscle fibers under excessively high $[Ca^{2+}]$ (Liu et al. 1993). Studies have indicated Li^+ can pass through the Na^+ channels (Hille 1971) and interfere with the metabolism of inositol phosphates by inhibiting the 1-phosphomonoesterase (Catt and Balla 1989). As a consequence, accumulated inositol 1,4,5-triphosphate may promote Ca^{2+} release from the SR. However, this may not be the major cause of contractile changes at low temperature because there is very little change in muscle contraction at 5°C when $[Na^+]_o$ is substituted by Li^+ up to 20%. Under this condition, the concentration of Li^+ (28.6 mM) is far above that (10 mM) which can cause accumulation of inositol 1,4,5-triphosphate in the cell. The importance of the Na^+-Ca^{2+} exchange in regulating $[Ca^{2+}]_i$ at low temperature is further illustrated by substitution of $[Na^+]_o$ with sucrose; changes in resting tension and muscle contraction at 5°C are even greater with sucrose substitution than with Li^+. Taken together, these results suggest that the Na^+-Ca^{2+} exchange mechanism is critically involved in the extrusion of excess Ca^{2+} from the cell at 5°C to maintain low $[Ca^{2+}]_i$ at low temperature.

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IV. EFFECTS OF NIFEDIPINE, VERAPAMIL AND Cd^{2+} ON CARDIAC CONTRACTIONS AT LOW TEMPERATURE IN RAT AND GROUND SQUIRREL

INTRODUCTION

Low temperature has profound effects on cardiac function. One important aspect is on the voltage-dependent Ca^{2+} channels in sarcolemmal membrane. Studies have indicated that the peak Ca^{2+} influx (I_{Ca}) through the voltage-dependent Ca^{2+} channels decreases markedly upon cooling in cardiac cells of non-hibernating species such as the rat and guinea pig (Mitchell et al. 1983; Cavalie et al. 1985, Liu et al. 1991). In a hibernating species (the European hedgehog), peak I_{Ca} amplitude does not change significantly at low temperature (Liu et al. 1991); but a recent study of another hibernating species, the Richardson's ground squirrel, has shown that the peak I_{Ca} decreased upon cooling in a manner similar to that seen in the guinea pig. The inactivation of I_{Ca} slows markedly under cooling, presumably leaving more time for Ca^{2+} entry at low temperature (Herve et al. 1992). This raises an interesting question: is the Ca^{2+} influx through voltage-dependent Ca^{2+} channels-even though at a reduced magnitude and slower rate-essential for cardiac excitation-contraction coupling at low temperature? Therefore we have studied cardiac contractile changes when the Ca^{2+} influx is blocked by Ca^{2+} channel blockers at low temperature.

The function of the voltage-dependent Ca^{2+} channels at low temperature is also relevant to the practice of clinical organ preservation. Recent studies have shown that a significant improvement of hypothermic preservation in kidney

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(Aureli et al. 1990; Cotterill et al. 1989), liver (Ar'Rajab et al. 1991; Chang et al. 1991) and lungs (Hachida and Morton 1989) can be realized if Ca^{2+} channel blockers (nifedipine and verapamil) are included in the preservation solution. In hypothermic heart preservation, however, the beneficial effect of Ca^{2+} channel blockers remains controversial (Yamamoto et al. 1983; Chan et al. 1990; Sukehiro et al. 1991). Evidence has suggested that Ca^{2+} channel blockers have little protective effect on heart function below 20°C (Fukunami and Hearse 1985). This is probably due to: a) the inhibitory effect of low temperature alone on Ca^{2+} channels (Mitchell et al. 1983; Cavalie et al. 1985; Liu et al. 1991) such that further addition of Ca^{2+} channel blockers elicits no additive effects; b) reduced sensitivity of Ca^{2+} channel proteins to the blockers at low temperature; and c) a use-dependent blockade of Ca^{2+} channel blockers since the heart is usually arrested while hypothermic.

The present study has been undertaken to provide the first report, to our knowledge, of cardiac contractile responses to Ca^{2+} channel blockers at low temperature. We have used a mammalian hibernating species, the Richardson's ground squirrels, in which cardiac cells maintain contractile function at as low as 5-0°C. We have also used rats as a comparison of non-hibernating species, since the mechanical (force-interval relationship) and electrical (transmembrane potentials) properties of rat heart at 37°C are similar to those of the Richardson's ground squirrels (Zhou et al. 1987, Zhou et al. 1991, Zhao, Dryden and Wang unpublished data). The effects of the organic blockers nifedipine and verapamil, and an inorganic blocker cadmium (Cd^{2+}), have been compared in both ground squirrels and rats. Since heart rate decreases markedly at low temperature, the contractile changes under different stimulus frequencies have also been compared.

MATERIALS AND METHODS

All experimental procedures reported herein received prior approval by the University of Alberta Animal Use Committee following the guidelines of the Canadian Council on Animal Care.

Muscle preparations and isometric contractions. Richardson's ground squirrels (*Spermophilus richardsonii*) were trapped in farm fields near Edmonton, Alberta, Canada. The animals were maintained individually with food and water ad libitum at 22°C and a 12:12 h light-dark photoperiod. Male Sprague-Dawley rats were maintained under the same conditions as the ground squirrels. Both species were sacrificed by decapitation. The hearts were rapidly excised and immersed in cold (4°C) Krebs-Henseleit solution which contained (in mM): 118 NaCl, 4.7 KCl, 1.6 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.0 $CaCl_2$ and 11 glucose, aerated with 95%O_2 and 5%CO_2 (pH 7.4). The right ventricular muscles were cut into pieces (1 mm x 5 mm) under a dissecting microscope (Vickers) and mounted vertically in 5 ml thermostated bath chambers. One end of a muscle was tied with 5-0 thread to the holder of an electrode and the other to a force transducer (Grass FT03C). Muscles were equilibrated at 37°C for 60 minutes and stretched until maximal contractions were reached under 1.0 Hz electrical stimulus with a WPI A310 Accupulser. The stimulus (5 ms in duration and 10 mA in intensity) frequencies at 37°C were 1.0 Hz and 0.2 Hz, while only 0.2 Hz was used at 15 and 5°C. For the experiments at lower temperatures, the bath temperature was then gradually decreased to 15 or 5°C within 40 minutes. The muscles were continuously stimulated at 0.2 Hz for another 60 minutes at the desired temperature before the isometric contractions were recorded with a Grass polygraph (model 7D). The data were also stored in an IBM/PC using CODAS, a data acquisition program (DATAQ Instruments Inc.), at a sampling rate of 1 kHz for later analysis. The contractile changes in the cardiac muscles of the Richardson's ground squirrels were recorded at 37, 15 and 5°C, while those of the rats were only recorded at 37 and 15°C.

Drug administration. Nifedipine, verapamil and Cd^{2+} ($CdCl_2$) were purchased from Sigma Chemical Company (St. Louise, MO, U.S.A.). Nifedipine was dissolved in dimethylsulfoxide (DMSO) in a series of stock solutions. Verapamil and Cd^{2+} were dissolved in double distilled water in a series of stock solutions. Both nifedipine and verapamil stock solutions were wrapped with aluminum foil since they are light sensitive. Cumulative dose-response curves for each of the three drugs were obtained separately in different muscles. The total volume change after addition of each drug was less than 1% of the bathing medium.

Analysis. Data were presented as means ± SE. The dosages for half-maximal inhibition (IC_{50}) of muscle contractions of the three drugs were calculated using the Hill equation. Multiple comparisons of IC_{50} values at different temperatures were made using ANOVA on an IBM/PC (SPSS/PC+). P values < 0.05 were considered significant.

RESULTS

Contractile changes in ventricular muscles of rats. Figure IV.1 shows the dose-response relationship of rat ventricular muscle contraction to nifedipine. When muscle was stimulated at 1.0 Hz at 37°C, the amplitude of contraction began to decrease after addition of 5×10^{-8} M nifedipine, and IC_{50} under this condition was 1.8 ± 0.6 μM (Table IV.1). When the stimulus frequency was decreased to 0.2 Hz, the dose-response curve shifted to the right, and the IC_{50} increased significantly ($p<0.05$) to 43 ± 5 μM, indicating the inhibitory effect of nifedipine is use-dependent. When temperature was decreased to 15°C, the IC_{50} at 0.2 Hz stimulus was not significantly altered (55 ± 12 μM).

The effect of verapamil on the contraction of rat ventricular muscle is shown in Figure IV.2. At 37°C, the IC_{50} was 1.8 ± 0.7 μM at 1.0 Hz and 75 ± 18 μM at 0.2 Hz, respectively (Table IV.1). When temperature was decreased to 15°C, the IC_{50} at 0.2 Hz did not change significantly and the dose-response curve was actually

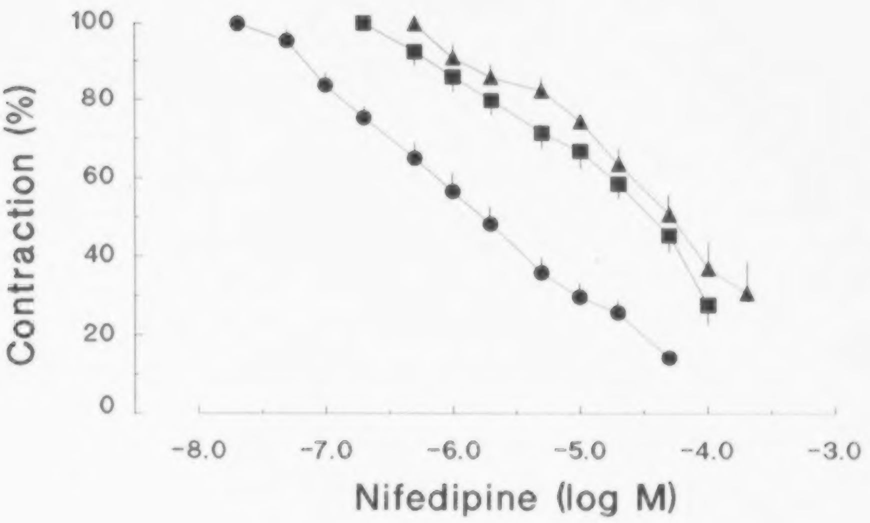

Figure IV.1. Effect of nifedipine on the contractions of rat ventricular muscles stimulated at 1.0 Hz at 37°C (filled circles), 0.2 Hz at 37°C (filled squares) and 0.2 Hz at 15°C (filled triangles). means±SE, n=8 in each group.

Table IV.1. The dosage (in μM) for half-maximum inhibition (IC_{50}) of cardiac muscle contractions by nifedipine, verapamil and cadmium (Cd^{2+}) in rats and active Richardson's ground squirrels (G.S.).

		37°C		15°C	5°C
		1.0 Hz	0.2 Hz	0.2 Hz	0.2 Hz
Rat	Nifedipine	1.8±0.6	43±5*	55±12*	n.d.
	Verapamil	1.8±0.7	75±18*	81±20*	n.d.
	Cd^{2+}	70±18	318±40*	40±5+	n.d.
G.S.	Nifedipine	1.3±0.2	126±15*	176±52*	n.d.
	Verapamil	5.8±1.0	5.6±1.4	35±8*+	119±30*+
	Cd^{2+}	328±56	658±90*	48±9*+	11±1*+

* Significantly different ($p<0.05$) from that at 37°C under 1.0 Hz stimulus.

+ Significantly different ($p<0.05$) from that at 37°C under 0.2 Hz stimulus.

n.d. Not determined.

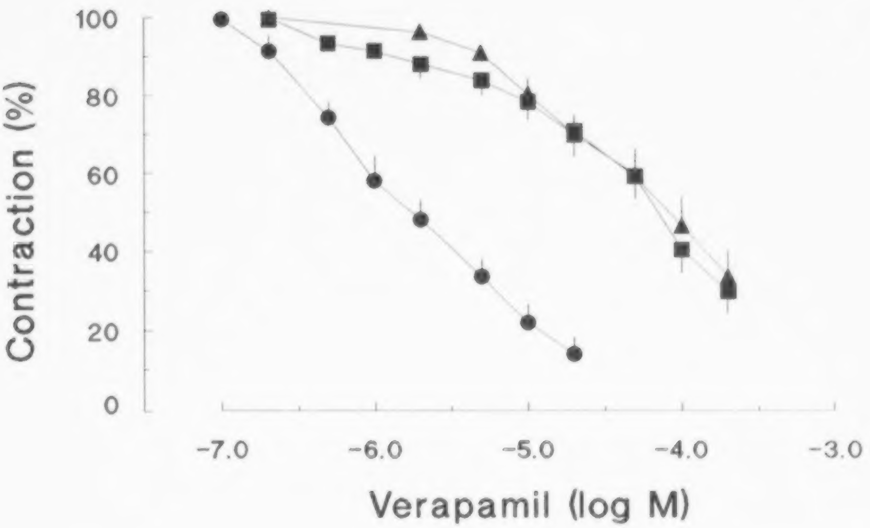

Figure IV.2. Effect of verapamil on the contractions of rat ventricular muscles stimulated at 1.0 Hz at 37°C (filled circles), 0.2 Hz at 37°C (filled squares), and 0.2 Hz at 15°C (filled triangles). means±SE, n=8 in each group.

superimposable with that at 37°C at 0.2 Hz stimulus.

The responses to Cd^{2+} were more complex. While a use-dependence was indicated by the rightward shift of dose-response curve at 37°C, two phases of contractile response to Cd^{2+} were observed at 15°C (Fig. IV.3). The IC_{50} was 70±18 and 318±40 μM, respectively, when stimulus frequency at 37°C was 1.0 and 0.2 Hz. At 15°C, the amplitude of contraction at 0.2 Hz stimulus began to decrease at 1.0 μM Cd^{2+}, which was similar to that at 37°C. At a Cd^{2+} concentration of 10 μM or greater, however, the amplitude of contraction decreased more markedly than that at 37°C. A determination of IC_{50} at 15°C is therefore of limited value, as it would be a hybrid of two processes, however, the empirical value was 40±5 μM.

Contractile changes in ventricular muscles of ground squirrels. The effects of nifedipine, verapamil and Cd^{2+} are shown in Figures IV.4, IV.5, and IV.6, respectively. A use-dependent effect was observed in nifedipine at 37°C (Fig.IV.4). When stimulus frequency was changed from 1.0 Hz to 0.2 Hz, the dose-response curve shifted to the right and IC_{50} increased from 1.3±0.2 μM to 126±15 μM. Further decrease of temperature to 15°C did not change the IC_{50} value significantly (Table IV.1).

The effect of verapamil did not show use-dependency at 37°C and the dose-response curves at 1.0 Hz and 0.2 Hz were superimposable (Fig.IV.5). When temperature was decreased from 37°C to 15 and 5°C, the sensitivity of muscle contraction to verapamil decreased and the IC_{50} under 0.2 Hz stimulus increased ($p<0.05$) from 5.6±1.4 μM to 35±8 μM and 119±12 μM, respectively (Fig.IV.5; Table IV.1).

As stimulus frequency was decreased from 1.0 Hz to 0.2 Hz, the IC_{50} of Cd^{2+} at 37°C increased ($p<0.05$) from 328±56 μM to 658±90 μM (Fig.IV.6;Table IV.1).

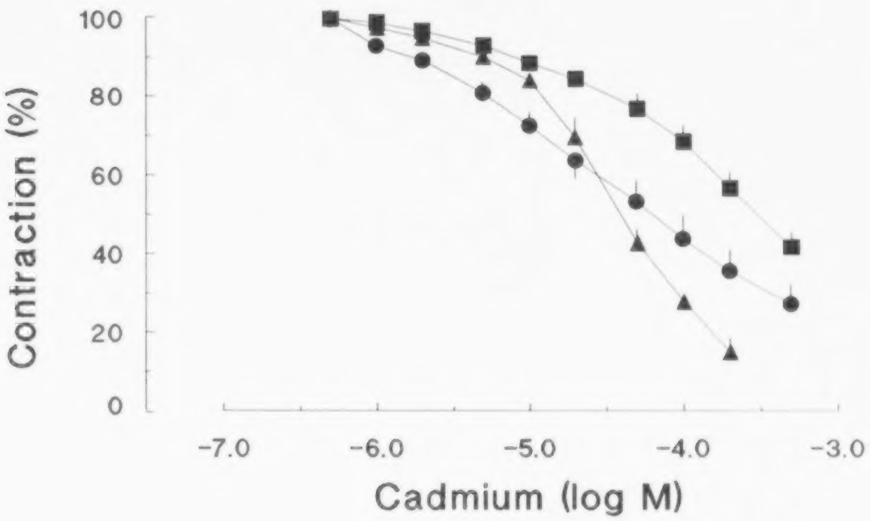

Figure IV.3. Effect of Cd^{2+} on the contractions of rat ventricular muscles stimulated at 1.0 Hz at 37°C (filled circles), 0.2 Hz at 37°C (filled squares), and 0.2 Hz at 15°C (filled triangles). means±SE, n=7-12 in each group

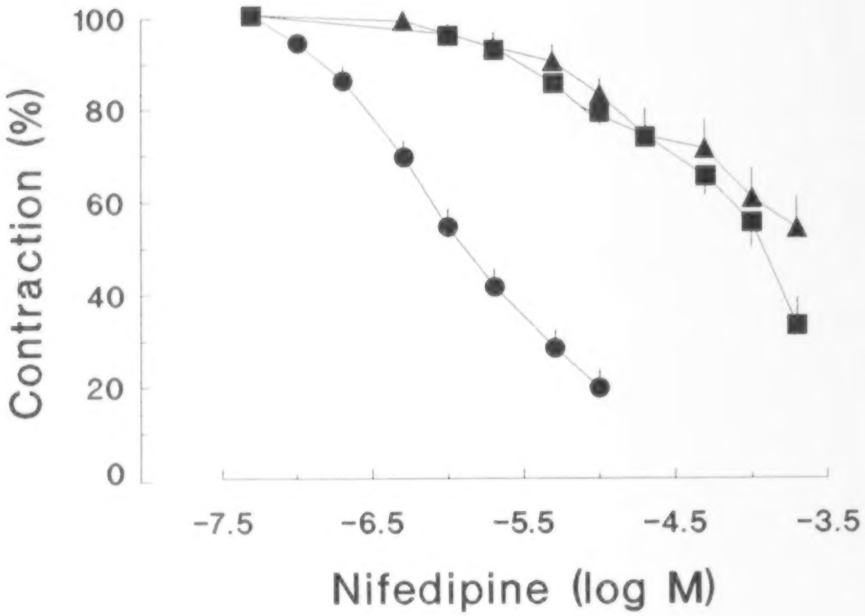

Figure IV.4. Effect of nifedipine on the contractions of ground squirrel ventricular muscles stimulated at 1.0 Hz at 37°C (filled circles), 0.2 Hz at 37°C (filled squares) and 0.2 Hz at 15°C (filled triangles). means±SE, n=7-12 in each group.

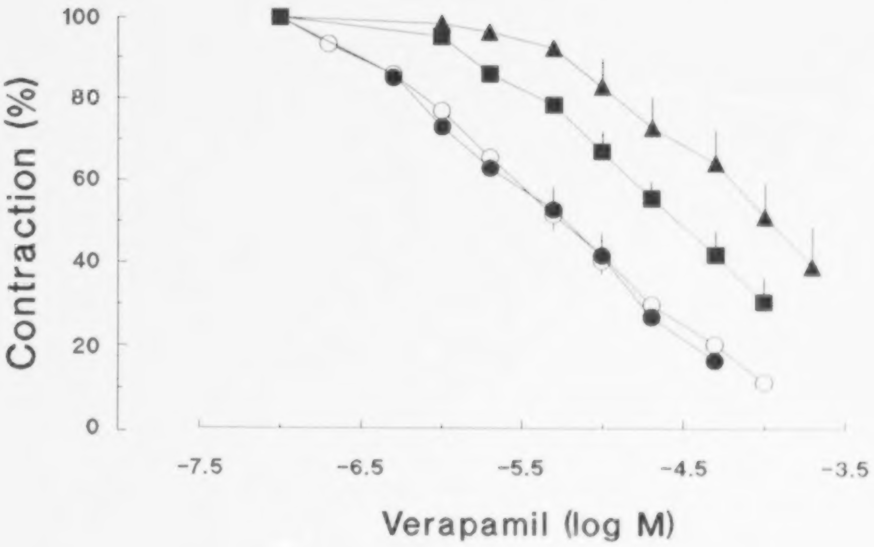

Figure IV.5. Effect of verapamil on the contractions of ground squirrel ventricular muscles stimulated at 1.0 Hz at 37°C (open circles), 0.2 Hz at 37°C (filled circles), 0.2 Hz at 15°C (filled squares), and 0.2 Hz at 5°C. means±SE, n=7-13 in each group.

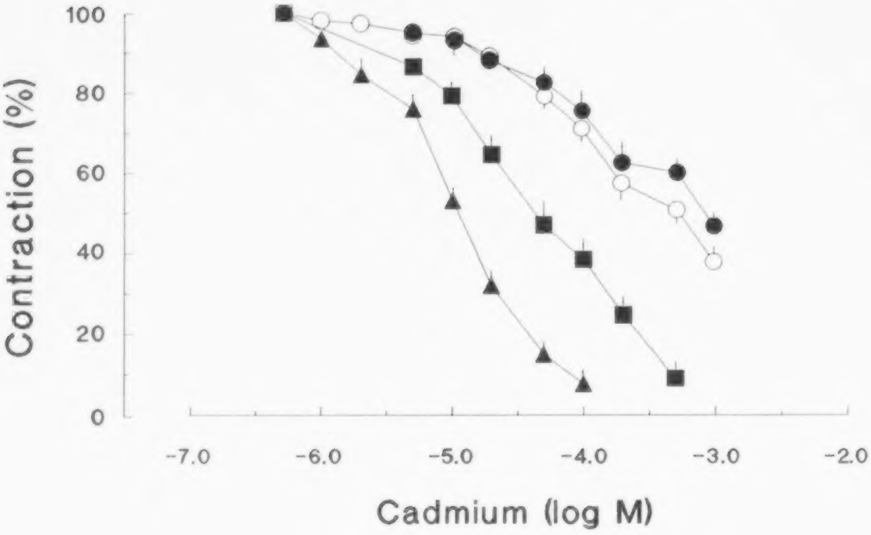

Figure IV.6. Effect of Cd^{2+} on the contractions of ground squirrel ventricular muscles stimulated at 1.0 Hz at 37°C (open circles), 0.2 Hz at 37°C (filled circles), 0.2 Hz at 15°C (filled squares), and 0.2 Hz at 5°C (filled triangles). means±SE, n=8-16 in each group.

In contrast to those observed after addition of nifedipine or verapamil, the sensitivity of muscle contraction to Cd^{2+} increased markedly at low temperatures.Similar to those observed in rat ventricular muscles, the contractile responses to Cd^{2+} in ground squirrels also showed two phases in dose-response curve at 15 and 5°C with a second steeper component when Cd^{2+} concentration was greater than 5-10 μM. The ventricular muscle contractions were more sensitive to Cd^{2+} at 5°C than at 15°C. The empirical values of IC_{50} were 48±9 μM and 11±1 μM, respectively, at 15 and 5°C (Table IV.1).

DISCUSSION

It has been well established that the L-type Ca^{2+} channels which mediate a long-lasting Ca^{2+} influx are predominant in cardiac ventricular cells (Tsien 1983; Bean 1989; Pelzer et al. 1990). These Ca^{2+} channels are inhibited by three distinct classes of organic blockers: dihydropyridines such as nifedipine, phenylalkylamines such as verapamil, and benzothiazepines such as diltiazem (Hondeghem and Katzung 1984; Hurwitz 1986). Radioligand binding studies indicate that these structurally different agents act at three separate but allosterically linked binding sites in the channel protein (Triggle and Janis 1987). A prominent feature of Ca^{2+} channel blockade by each of these blockers is use-dependence, in which an increase in stimulus frequency will induce a greater degree of inhibitory effect on the Ca^{2+} channels (Hondeghem and Katzung 1984; Hurwitz 1986; Triggle and Janis 1987). This suggests that ligand affinity for the Ca^{2+} channels varies depending on different states of the channel protein. The Ca^{2+} channel blockers have little affinity when the channel protein is in the resting state, while they interact to a great degree with the channel protein when the latter is in an activated or inactivated state (Hurwitz 1986; Triggle and Janis 1987). The off-rate constants for ligand dissociation are such that at physiological frequencies of stimulation, there is a sufficient accumulation of inhibited channels to affect the contractile response.

The L-type Ca^{2+} channels in ventricular cells are also sensitive to many inorganic divalent and polyvalent cations such as La^{3+}, Cd^{2+}, Co^{2+}, Mn^{2+} (Hagiwara and Byerly 1981; Lansman et al. 1986). However, the blockade mechanisms of these inorganic cations are different from those of organic agents. Studies have indicated that these cations compete with Ca^{2+} for multiple binding sites within the channel pore (Lansman et al. 1986; Tsien et al. 1987; Rosenberg and Chen 1991). Use-dependent blockade of L-type Ca^{2+} channels is also observed with these inorganic cations (Lee and Tsien 1983; Lansman et al. 1986).

In the present study, ventricular contractile responses to Ca^{2+} channel blockers at low temperature have been investigated, and compared between a hibernating species, the Richardson's ground squirrels, and a non-hibernating species, the rats. Both organic (nifedipine and verapamil) and inorganic (Cd^{2+}) Ca^{2+} channel blockers have been used because of their different blockade sites. In rats, a use-dependent inhibitory effect on contraction is observed at 37°C with nifedipine and verapamil. When stimulus frequency is decreased from 1.0 Hz to 0.2 Hz at 37°C, the dose-response curves for these two agents shift to the right and their IC_{50} values increase significantly ($p<0.05$). These results are consistent with use-dependent blockade of the Ca^{2+} channels by these agents observed in various studies (Hurwitz 1986; Triggle and Janis 1987). When ventricular muscles are stimulated with 0.2 Hz at 15°C, the IC_{50} values and dose-response curves of both nifedipine and verapamil do not change significantly from those seen at 37°C under same stimulus frequency. Since heart rate decreases markedly upon cooling, and the heart is usually arrested in the cardioplegic solution during hypothermic preservation, the loss of protective effects of these organic Ca^{2+} channel blockers may explained at least in part by their characteristic use-dependence.

A similar use-dependent effect of Cd^{2+} is also observed in rat ventricular muscles at 37°C. The two phases in dose-response curve at 15°C suggest that the increased inhibitory effect of Cd^{2+} on muscle contraction at low temperature may not be solely due to reduction of the Ca^{2+} influx across the sarcolemmal membrane. It may also be due to its intracellular effects (Foulkes 1986). Studies of non-cardiac cells have indicated that Cd^{2+} can enter cells through Ca^{2+} channels or an anion exchanger (Blazka and Shaikh 1991; Lou et al. 1991). Although there is no direct evidence that Cd^{2+} can enter cardiac cells (Kopp 1986), Cd^{2+} has been shown to inhibit cardiac mitochondrial respiration *in vitro* (Kisling et al. 1987). However, the increased inhibitory effect of Cd^{2+} at low temperature may occur mainly on the Ca^{2+} channels in the sarcolemmal membrane since the Cd^{2+} influx is decreased by cooling (Garty et al. 1986; Blazka and Shaikh 1991).

In the ground squirrel, a use-dependent effect on muscle contraction at 37°C is observed with nifedipine and Cd^{2+}, but not with verapamil. The contractile responses to nifedipine are similar to those seen in the rat (compare Figs. IV.1 and IV.4). Lowering the temperature reduces sensitivity to verapamil. Similar to results seen in the rat but observed even greater clarity, lowering the temperature from 37°C to 15 and 5°C results in increased sensitivity of muscle contraction to Cd^{2+} inhibition (Table IV.1 and Fig.IV.6).

Since nifedipine and verapamil interact with their own binding sites in the channel protein while Cd^{2+} competes with Ca^{2+} for common multiple binding sites within the channel pore, lowering the temperature may have different effects of their blockade actions on the Ca^{2+} channels. Evidence has indicated that the maximal high affinity binding (B_{max}) of (+)[^{3}H]PN 200-110, a high-affinity dihydropyridine ligand, does not change significantly in rat heart under hypothermic (22°C) condition (Gu et al. 1988). In guinea pig ventricle, however, the B_{max} of [^{3}H]nitrendipine decreases significantly when temperature is lowered from 37°C to 0°C (Rampe et al. 1987). At low temperature, the rate of configurational changes in channel proteins may be decreased, such that the affinity for the ligand binding may be altered (Boles et al. 1984; Howlett and Gordon 1990). These changes in the binding sites may account for the decreased sensitivity of cardiac contraction to verapamil at low temperature observed in ground squirrels. Alternatively, the cardiac cells of Richardson's ground squirrels (but not rats) may be less dependent, but never independent, on extracellular Ca^{2+} for excitation-contraction coupling when temperature is progressively decreased. The changes in the binding sites of channel proteins may also account for increased sensitivity of cardiac muscle to Cd^{2+} at low temperature in both rats and ground squirrels. Since the Ca^{2+} influx through the Ca^{2+} channels decreases markedly upon cooling in both species (Mitchell et al. 1983; Liu et al. 1991; Herve et al. 1992), less Cd^{2+} is required to block the Ca^{2+} influx at low temperature.

Taken together, use-dependent blockade and the decrease in muscle sensitivity to Ca^{2+} channel blockers upon cooling may explain the minimum protective effect of organic Ca^{2+} channel blockers at low temperature observed in previous studies. Further studies are needed to verify whether the increased sensitivity of muscle contraction to Cd^{2+} at low temperature, as observed in both hibernating and non-hibernating species, is due to the actions of Cd^{2+} on Ca^{2+} channels, or of Cd^{2+} on other intracellular mechanisms, or both. It may also be of interest to test whether Cd^{2+}, due to its increased inhibitory effects at low temperature, can enhance functional recovery of the heart after hypothermic preservation.

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V. EFFECTS OF TEMPERATURE AND pH ON CARDIAC MYOFILAMENT Ca^{2+} SENSITIVITY IN RAT AND GROUND SQUIRREL [4]

INTRODUCTION

Survival in hypothermia and hibernation requires well-maintained cardiac function in order to overcome the marked increase in peripheral resistance at low body temperature (T_b) (Lyman and O'Brien 1960; Wang 1988). Previously we have demonstrated that the force of electrically-paced cardiac contraction is significantly greater in papillary muscles isolated from hibernating ground squirrels than those from active ground squirrels (Zhou et al. 1991). This marked seasonal difference is evident at temperatures characteristic of both the euthermic (37°C) and hibernating state (7°C), indicating it is not simply a temperature effect (Zhou et al. 1991). The enhanced cardiac contractility observed during the hibernating phase is likely an adaptation to ensure adequate tissue perfusion despite the increased peripheral resistance at low T_b.

The physiological/biochemical basis for the increased cardiac contractility during the hibernating state is currently unknown. It is generally expected, however, that seasonal modifications in the processes governing excitation-contraction coupling are involved. Evidence indicates that the transsarcolemmal Ca^{2+} influx through voltage-dependent Ca^{2+} channels decreases during the hibernation phase in the Asiatic chipmunk (Kondo and Shibata 1984; Kondo 1986) and that the heart depends more on Ca^{2+} released from the sarcoplasmic reticulum (SR) than on the extracellular Ca^{2+} influx. In support of this observation, we have shown that contractility of the hibernating ground squirrel heart is only minimally sensitive to variations of extracellular Ca^{2+} concentration between 0.1 to 5 mM over several hours of exposure (Zhou et al. 1991). Further, a significant increase in cardiac SR Ca^{2+} uptake has also been observed in the hibernating state when compared to the active state (Belke et al. 1987; Belke et al. 1991); this increase may result in a greater amount of SR Ca^{2+} release during excitation, leading to enhanced cardiac contraction. However, since contraction is initiated by the binding of Ca^{2+} to contractile proteins (Thompson et al. 1990), an increase in the Ca^{2+} sensitivity of the myofilaments during hibernation may also explain the observed increase in cardiac contractility. The aim of the present study is to document seasonal shift in myofilament Ca^{2+} sensitivity which might occur in conjunction with hibernation.

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[4] A version of this chapter has been published: Liu, B., Wang, L. C. H. and Belke, D. D. (1993) Effects of temperature and pH on cardiac myofilament Ca^{2+} sensitivity in rat and ground squirrel. *Am. J. Physiol.* 264, R104-R108.

Mechanically- or chemically-skinned muscle has been used extensively to study the effect of temperature on the Ca^{2+} sensitivity of myofilaments in vertebrates. Results to date are quite varied depending on the species involved and the tissues used. For example, an increased myofilament Ca^{2+} sensitivity with decreased temperature has been observed in canine Purkinje fibers (Fabiato 1985) and in the skeletal muscles of the frog, toad and rat (Godt and Lindley 1982; Stephenson and Williams 1985). In contrast, a decrease in myofilament Ca^{2+} sensitivity with decreased temperature has been seen in cardiac ventricular muscles of the frog, rat, rabbit, and guinea pig (Harrison and Bers 1989a; Harrison and Bers 1990). Whether the cardiac myofilament Ca^{2+} sensitivity in ground squirrels follows the former trend or whether it shows a seasonal reversal between the two trends, are both open possibilities. Because temperature changes result in a shift in intracellular pH (pH_i) and changes in pH affect Ca^{2+} binding to troponin C (Thompson et al. 1990), the effects of both temperature and pH have been examined in the present study. Myofilament Ca^{2+} sensitivity has been examined at pH 7.00 for comparison with previous studies which were conducted at a static pH and under changing pH to reflect the physiological myocardial pH_i occurring during hibernation (Malan et al. 1985).

To provide an internal control, the skinned papillary muscles of rats have been used to verify both our methodology and the standard trend typically seen in a non-hibernating species.

MATERIALS AND METHODS

All experimental procedures reported herein received prior approval by the University of Alberta Animal Use Committee following the guidelines of the Canadian Council on Animal Care.

Muscle preparations. Richardson's ground squirrels (*Spermophilus richardsonii*) were trapped in farm fields near Edmonton, Alberta, Canada. The animals were maintained individually with food and water ad libitum at 22°C and 12:12 h light-dark photoperiod. The average T_b of the active ground squirrels was 37.7±0.1°C. When the animals reached the hibernating phase of their endogenous cycle as characterized by having achieved rapid weight gain, they were transferred to a cold room (4°C and 2:22 h light-dark) to facilitate hibernation. The average T_b of the hibernating ground squirrels used in this study was 4.0±0.4°C. Male Sprague-Dawley rats were obtained from the Biological Sciences Laboratory Animal Services on campus and kept under the same conditions as the active squirrels. Both species were killed by decapitation. The hearts were rapidly excised and immersed in cold (4°C) Krebs-Henseleit solution which contained (in mM): 118 NaCl, 4.7 KCl, 1.6 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 2.5 $CaCl_2$ and 11.0 glucose, gassed with 95% O_2 and 5% CO_2 (pH 7.4 at 25°C). Three right ventricular papillary muscles (0.2-0.45 mm in diameter) were excised under a dissecting microscope (Vickers) and mounted vertically in 5 ml thermostated chambers. The ventricular base of each muscle was tied with 5-0 thread to the holder of an electrode. A loop of suture thread was made in the tendinous end of the muscle and attached to the extended stainless steel arm of a force transducer (Grass FT03C).

The muscle was equilibrated and stimulated with 0.5 Hz at 25°C for 60 minutes. The stimulus in a constant current mode was used with 10 ms in duration and 50% above the threshold. During this period, the muscle was gradually stretched to a length at which the maximal contraction was achieved. The muscle was then incubated at 25°C for 40 minutes in a Ca^{2+}-ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) relaxation solution containing 2% vol/vol Triton X-100. The relaxation solution was essentially Ca^{2+}-free (pCa^{2+}, $-\log_{10}[Ca^{2+}]$ 9.0), and had a pH value of 7.00. The composition of the Ca^{2+}-EGTA solution, used to determine the myofilament Ca^{2+} sensitivity, was (in mM): 100 KCl, 5.0 $MgCl_2$, 10.0 EGTA, 10.0 3-(N-morpholino)propanesulfonic acid (MOPS), 5.0 Na_2-ATP, 10.0 Na_2-creatine phosphate, 15 unit/ml creatine phosphokinase and various Ca^{2+}. After the muscle was skinned with Triton X-100 (Harrison and Bers 1989a), the solution in the bath chamber was changed by quick draining and replacing with fresh Ca^{2+}-EGTA relaxation solution without Triton X-100 at the same temperature. The pH of each Ca^{2+}-EGTA solution was adjusted with KOH to the desired value (7.00, 7.20 or 7.40) at the various test temperatures (25, 15, or 5°C). Once the steady-state tension was achieved, the accumulative tension development of the skinned muscle in different Ca^{2+}-EGTA solutions was recorded with a polygraph (Grass Model 7E) at a paper speed of 2.5 mm/min. The maximal tension (F_{max}) in the optimal Ca^{2+}-EGTA solution was measured at 5° intervals between 30 and 5°C.

Calculation of free Ca^{2+} concentration ($[Ca^{2+}]$). A computer program based on the equations presented by Fabiato and Fabiato (1979) was used to calculate the $[Ca^{2+}]$ in each Ca^{2+}-EGTA solution. Corrections for proton and the Ca^{2+} association constants of EGTA at different temperatures and ionic strengths were based on the report of Harrison and Bers (1989b). The delta H values in the Van't Hoff equation were obtained from Martell and Smith (1974) for EGTA, and from Smith and Martell (1974) and Christensen and Izatt (1983) for ATP.

Statistics. The data are presented as means ± SE. Multiple comparisons of the $[Ca^{2+}]$ for half maximal tension (pCa^{2+}_{50}) at different temperatures in the three groups as well as the F_{max} in the active and hibernating ground squirrels were made using an Analysis of Variance test (SPSS/PC+), performed on an IBM PC. The Student's *t* test was used to compare the pCa^{2+}_{50} and F_{max} between the active and the hibernating squirrels at each temperature. P values < 0.05 were considered significant.

RESULTS

Temperature dependence of myofilament Ca^{2+} sensitivity. Figure V.1 shows the effect of temperature on the tension-pCa^{2+} relationship at pH 7.00 in papillary muscles from rats and ground squirrels. In rat papillary muscles at 25°C (Fig.V.1A), the muscle tension started to increase in pCa^{2+} 7.00 and reached its maximum at pCa^{2+} 5.19. Further increases in $[Ca^{2+}]$ depressed muscle tension. When temperature was decreased from 25°C to 15 and 5°C, the tension-pCa^{2+} curve shifted to the right, to higher $[Ca^{2+}]$. The pCa^{2+}_{50} was calculated using the Hill equation:

$$F/F_{max} = K_{app}[Ca^{2+}]^n/(1+K_{app}[Ca^{2+}]^n)$$

where F is the steady-state tension, K_{app} is an apparent affinity constant and n is the Hill coefficient (Harrison and Bers 1989a). In rat papillary muscles, the pCa^{2+}_{50} was 6.10 ± 0.04 at 25°C and decreased significantly ($p<0.05$) to 5.82 ± 0.05 at 15°C, and 5.59 ± 0.04 at 5°C, respectively (Table V.1).

In papillary muscles from active ground squirrels (Fig.V.1B), the myofilaments were more sensitive to Ca^{2+} than those from the rats in low $[Ca^{2+}]$ ($pCa^{2+}>6.00$) at 25 and 15°C. The pCa^{2+} values for F_{max}, however, were the same as those for rats at the same temperatures from 25 to 5°C. A significant shift of tension-pCa^{2+} curve to higher $[Ca^{2+}]$ at low temperatures was also observed in the active ground squirrel. The pCa^{2+}_{50} decreased significantly ($p<0.05$) from 6.43 ± 0.04 at 25°C to 6.00 ± 0.03 at 15°C, and 5.56 ± 0.07 at 5°C,

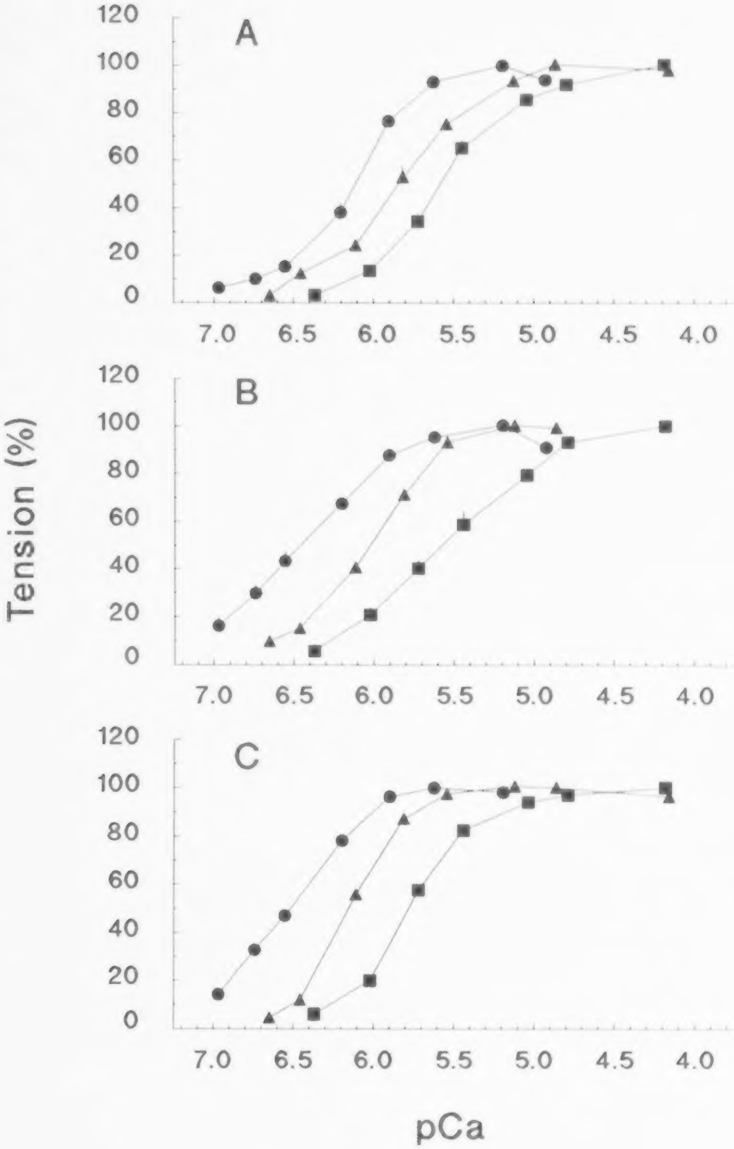

Figure V.1. Tension-pCa^{2+} relationship at pH 7.00 in chemically-skinned papillary muscles from rats (A), active (B) and hibernating (C) ground squirrels at 25°C (circles), 15°C (triangles), and 5°C (squares). means±SE, n=10-16 in each group.

Table V.1. Ca^{2+} concentration for half-maximal tension ($pCa^{2+}{}_{50}$), and Hill coefficient for skinned papillary muscles of rats and ground squirrels (G.S.) at 25°, 15°, and 5°C, pH 7.00.

	$pCa^{2+}{}_{50}$			Hill Coefficient		
	25°C	15°C	5°C	25°C	15°C	5°C
Rat	6.10±0.04*	5.82±0.05*	5.59±0.04*	2.07±0.18	1.97±0.05	1.80±0.16
	(6.30-5.79)	(6.16-5.60)	(5.85-5.37)			
n	12	11	10			
G.S.						
Active	6.43±0.04*	6.00±0.03*	5.56±0.07*	1.64±0.15	2.02±0.14	1.41±0.12
	(6.79-6.26)	(6.26-5.81)	(5.89-5.09)			
n	16	12	12			
Hibernating	6.50±0.02*	6.14±0.03^{*+}	5.80±0.01^{*+}	1.83±0.10	2.68±0.11^{+}	2.11±0.11^{+}
	(6.59-6.46)	(6.24-6.08)	(5.85-5.76)			
n	12	14	12			

Values are means ± SE. n, number of muscles. Range is given in parentheses. Significant difference ($p<0.05$) * among three temperatures, and $^{+}$ between active and hibernating ground squirrels at same temperature.

respectively (Table V.1).

At 25°C, the tension-pCa^{2+} curve of the hibernating ground squirrels (Fig.V.1C) was similar to that of the active ground squirrels. No significant difference was observed in the pCa^{2+}_{50} values between the two groups (Table V.1). At 15 and 5°C, the threshold pCa^{2+} for tension development was 6.66 and 6.37, respectively. The threshold pCa^{2+} for tension development was similar for hibernating and active ground squirrels at these temperatures. As the $[Ca^{2+}]$ was increased to the intermediate range, however, the muscle tension was higher in the hibernating ground squirrel. The Hill coefficient (n) was significantly higher ($p<0.05$) for the hibernating than the active group at these temperatures (Table V.1). The pCa^{2+}_{50} for hibernating ground squirrels was 6.14 at 15°C and 5.80 at 5°C, being significantly different ($p<0.05$) from that obtained from active ground squirrels at the same temperatures (Table V.1). The F_{max} values at 15 and 5°C in the hibernating ground squirrels were reached at the same $[Ca^{2+}]$ as in the active squirrels.

F_{max} of myofilaments at different temperatures. The F_{max} from 30 to 5°C was compared between the active and hibernating ground squirrels at pH 7.00 (Fig.V.2). In active ground squirrels, F_{max} increased as temperature decreased from 30°C, and reached the maximal value at 15°C. The maximal value for F_{max} in the hibernating ground squirrel was obtained at 20°C. No significant difference, however, was observed between the two groups at all temperatures tested.

Effects of pH change on myofilament Ca^{2+} sensitivity at 25°C and 5°C. Because pH_i increases with cooling in the cardiac cells of hibernators with a temperature coefficient about -0.013 (Malan et al. 1985), the skinned muscle tensions in various $[Ca^{2+}]$ at 25°C with pH 7.20 was compared with those at 5°C with pH 7.40 in the active ground squirrels. It can be seen in Figure V.3 that

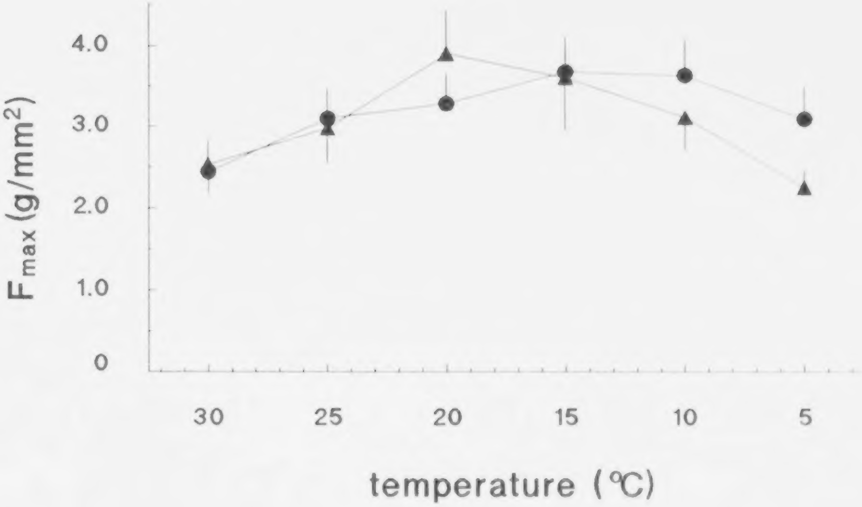

Figure V.2. Maximal tension (F_{max}) in Ca^{2+}-EGTA solution at pH 7.00 at each temperature from 30 to 5°C in active (n=12, circles) and hibernating (n=14, triangles) ground squirrels. means±SE.

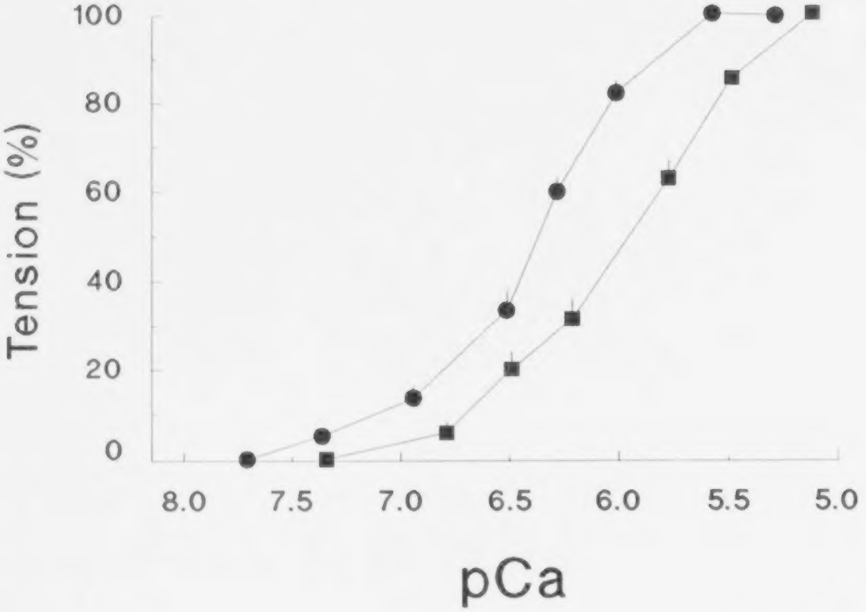

Figure V.3. Tension-pCa^{2+} relationship in chemically-skinned papillary muscles from active ground squirrels at 25°C with pH 7.20 (circles) and at 5°C with pH 7.40 (squares). means±SE, n=14 at 25°C and n=9 at 5°C.

even at physiological pH_i, the tension-pCa^{2+} curve shifted to higher $[Ca^{2+}]$ at low temperature, and the pCa^{2+}_{50} (5.88±0.06) at 5°C and pH 7.40 was significantly lower ($p<0.05$) than that (6.40±0.03) at 25°C and pH 7.20.

The myofilament Ca^{2+} sensitivity between the active and hibernating ground squirrels was also compared at 5°C with pH 7.40 (Fig.V.4). The threshold $[Ca^{2+}]$ for muscle tension development was about 1.62×10^{-7} M in both groups. As the $[Ca^{2+}]$ increased above 4.17×10^{-7} M (pCa^{2+} 6.38), however, the muscle tension in the hibernating group was about 20% higher than that in the active animals at the same $[Ca^{2+}]$. The pCa^{2+}_{50} (6.13±0.05) of the hibernating squirrels was significantly higher ($p<0.05$) than that (5.88±0.06) of the active squirrels. The calculated Hill coefficient was also significantly higher ($p<0.05$) for the hibernating group in comparison with the active group at this temperature and pH (Hibernating 2.14±0.29, Active 1.49±0.10).

DISCUSSION

In the present study, the effect of temperature on cardiac myofilament Ca^{2+} sensitivity has been compared between rats and ground squirrels. In rat papillary muscles at 25°C, the pCa^{2+}_{50} value and the optimal $[Ca^{2+}]$ for F_{max} at pH 7.00 are similar to the values reported for single rat ventricular cells at 22°C (Fabiato and Fabiato 1978). After the tension reaches its maximum, a further increase in $[Ca^{2+}]$ depressed muscle tension, which is also similar to the results seen in single ventricular cells at 22°C (Fabiato and Fabiato 1978). The depressed tension development in supra-optimal $[Ca^{2+}]$ may be due to a reduction of myofibrillar ATPase activity (Portzehl et al. 1969). When temperature is decreased from 25 to 5°C, the Ca^{2+} sensitivity of the rat myofilaments decreases and the pCa^{2+}_{50} at pH 7.00 increases by 0.025 pCa^{2+} units/°C on average, which is consistent with an earlier study on rats which shows an increment of 0.023 pCa^{2+} units/°C in pCa^{2+}_{50} (from 29 to 1°C) (Harrison and Bers 1990). From this,

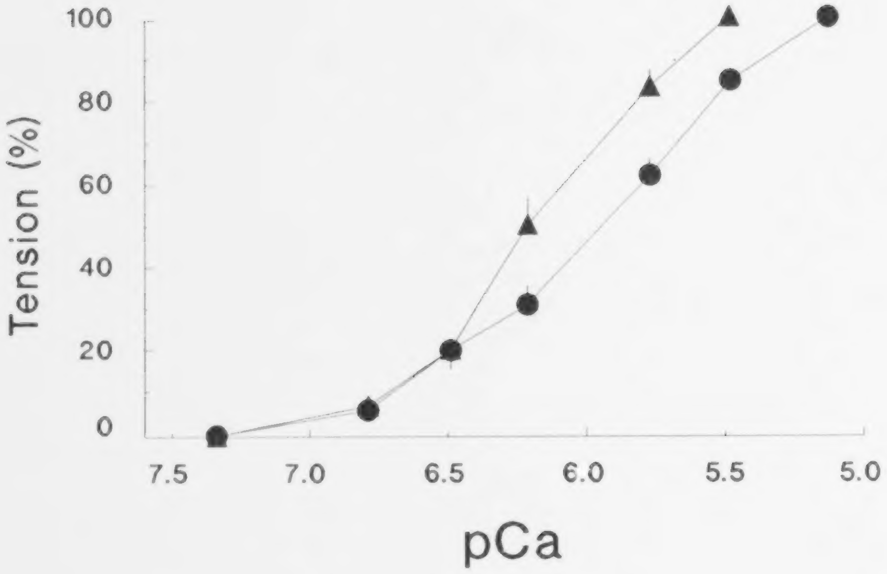

Figure V.4. Tension-pCa^{2+} relationship in chemically-skinned papillary muscles from active (circles) and hibernating (triangles) ground squirrels at 5°C with pH 7.40. means±SE, n=9 in each group.

we conclude that the methodologies employed in our present study are directly comparable to those employed by other investigators.

In comparison with results seen in the rat, the cardiac myofilaments of both active and hibernating ground squirrels are more sensitive to Ca^{2+} at 25°C and pH 7.00 when $[Ca^{2+}]$ is below pCa^{2+} 6.00. A previous study with rat ventricular muscle shows that F_{max} at pH 7.00 decreases significantly at 8 and 1°C, being 30% and 3.3% of that at 29°C, respectively (Harrison and Bers 1990). In our present study on active and hibernating ground squirrels the maximal value of F_{max} at pH 7.00 in optimal $[Ca^{2+}]$ is found at 15 and 20°C, respectively. The F_{max} at 30°C is only about 66% of the respective maximal values in both groups. Thus, significant species difference exists in the optimal temperature for cardiac muscle F_{max}; however, cardiac myofilament Ca^{2+} sensitivity decreases significantly at low temperature in both active and hibernating ground squirrels, which is consistent with results in rat, rabbit and guinea pig (Harrison and Bers 1989a; Harrison and Bers 1990).

The higher Hill coefficient, coupled with a greater Ca^{2+} sensitivity at lower temperatures (15 and 5°C) in the hibernating group, suggest a higher efficiency in Ca^{2+} binding to troponin C. While this aids in increasing cardiac contractility at low temperatures, it is difficult to assess exactly how great an increase in contractile potential this represents. It is therefore unlikely that this change alone can account for the increase in contractility observed in the hibernating state, especially since the increase in contractility is observed at all temperatures, not just below 15°C.

In addition to the temperature effect, the influence of pH on myofilament Ca^{2+} sensitivity has also been studied at 25 and 5°C in both active and hibernating ground squirrels. It has been demonstrated that the heart and liver are exceptions to the marked tissue acidification prevailing during hibernation

(Malan et al. 1985). The change of pH_i in cardiac muscle during hibernation seems to follow "alpha-stat" regulation, which is manifested by an increase in pH_i as T_b decreases; but the dissociation ratio of the protein imidazole buffer groups remains fairly constant. The pH_i of heart is about 7.36 in the European hamster during hibernation at T_b of 10°C, but about 7.04 in the euthermic state at T_b of 37°C (Malan et al. 1985). The temperature coefficient of pH is approximately -0.013±0.001 for cardiac cells from the European hamsters. Because there is no direct measurement of pH_i in ground squirrel heart, we have assumed that the pH_i of ground squirrel cardiac cells changes in a similar way with temperature as in the European hamster. Therefore, the physiological pH_i for cardiac cells is about 7.20 at 25°C and 7.40 at 5°C. Even taking these conditions into consideration, the myofilament Ca^{2+} sensitivity in the active ground squirrels is still significantly less at 5°C (pH 7.40) (Fig.V.3) than that at 25°C (pH 7.20).

Because the contractility of the electrically-paced, intact papillary muscle increases significantly in hibernating vs. active ground squirrels (Zhou et al. 1991), we have tried to determine if this may be due to a seasonal difference in myofilament Ca^{2+} sensitivity. At 25°C, the tension-pCa^{2+} curve is similar and no significant difference is observed in pCa^{2+}_{50} at pH 7.00 between active and hibernating ground squirrels (Fig.V.1). At 15 and 5°C with pH 7.00, the threshold $[Ca^{2+}]$ for initiation of muscle tension is similar between the two groups. At the intermediate $[Ca^{2+}]$ range (pCa^{2+} 6.20-5.80 at 15°C, and pCa^{2+} 5.70-5.10 at 5°C), a higher muscle tension in hibernating animals is observed (Fig.V.1). At physiological pH_i (pH 7.40) at 5°C, a higher pCa^{2+}_{50} is also observed in hibernating ground squirrel vs. the active animals. In comparison with the pCa^{2+}_{50} values at physiological pH_i conditions (at 25 and 5°C), the pCa^{2+}_{50} around 6.13 at 5°C with pH 7.40 in the hibernating squirrels is still lower than that (pCa^{2+} range 6.40-6.50) at 25°C with pH 7.20. This suggests that the relatively higher Ca^{2+} sensitivity of the cardiac myofilaments in the hibernating ground squirrels may only partially contribute to the increased contractility in hibernation.

In summary, myofilament Ca^{2+} sensitivity, measured in skinned cardiac muscles under physiological pH_i, decreases with temperature in both active and hibernating ground squirrels. This suggests that the enhanced contractility in intact cardiac muscle observed at low temperature cannot be explained by any favourable change in myofilament Ca^{2+} sensitivity; rather, more Ca^{2+} may be required at low temperature to initiate mechanical contraction. The observed greater Ca^{2+} sensitivity and greater degree of co-operativity in the binding of Ca^{2+} to troponin C, as evidenced by the higher Hill coefficient in the hibernating group, may enhance its cardiac contractility, especially at lower temperatures. Such extrapolations from skinned fibers to intact muscle should be viewed with caution, due to the differences in the media around the contractile proteins: cellular factors such as naturally-soluble Ca^{2+} sensitisers and other proteins which are present in the intact state are lost when the muscle fiber is skinned. Such factors may contribute to increased contractility during hibernation in an intact preparation while they may not be detected in skinned muscle preparations. Thus, although enhanced contractility at low temperature is most likely due to increased Ca^{2+} release by the SR, since the Ca^{2+} influx through voltage-dependent Ca^{2+} channels is reduced (Mitchell et al. 1983; Cavalie et al. 1985; Herve et al. 1992) or relatively constant (Liu et al. 1991) at low temperature, the contribution of altered myofibril Ca^{2+} sensitivity cannot be eliminated entirely.

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VI. Ca^{2+} UPTAKE BY CARDIAC SARCOPLASMIC RETICULUM AT LOW TEMPERATURE IN RAT AND GROUND SQUIRREL

INTRODUCTION

In mammalian cardiac cells, Ca^{2+} uptake by the sarcoplasmic reticulum (SR) plays a crucial role in muscle relaxation (Bers 1991; Lytton and MacLennan 1991). During a normal cardiac contraction-relaxation cycle, Ca^{2+} influx across the sarcolemmal membrane triggers the rapid release of Ca^{2+} from SR to initiate mechanical contraction (Callewaert 1992; Stern and Lakatta 1992). Subsequent activity of SR Ca^{2+}-ATPase transports a large fraction of the released Ca^{2+} back into the SR lumen, resulting in a rapid decrease of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and muscle relaxation. The resting diastolic $[Ca^{2+}]_i$ is restored by this SR Ca^{2+} uptake, together with Ca^{2+} extrusion by the Na^+-Ca^{2+} exchanger in sarcolemmal membrane (Bers et al. 1992).

In order to understand how the cardiac cells of mammalian hibernators can maintain contractile function at low temperature without serious arrhythmias, the temperature effects on SR Ca^{2+} uptake need to be investigated. In a hibernating species, the Richardson's ground squirrel, the relationship of the rate of SR Ca^{2+} uptake to temperature is curvilinear when examined in the Arrhenius plot (Belke et al. 1987). A significant increase in the rate of SR Ca^{2+} uptake is observed in the hibernating state compared with that in the active state (Belke et al. 1987; Belke et al. 1991). This has been referred to as a special physiological adaptation when the animal enters into deep hibernation (Wang 1988). In non-hibernators, a linear relationship of Ca^{2+} uptake to temperature in the Arrhenius plot is observed in isolated SR from dog and rabbit hearts

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(Suko 1973). It seems that the function of cardiac SR Ca^{2+} uptake at low temperature is different between the hibernators and non-hibernators. However, this observed difference may be due to methodologies used in different studies. To further document temperature effects on cardiac SR Ca^{2+} uptake, the present study has therefore been conducted to compare the Ca^{2+} uptake by isolated SR between a hibernating species, the Richardson's ground squirrel, and a non-hibernating species, the rat, under the same experimental conditions. Since SR Ca^{2+} uptake depends on SR Ca^{2+}-ATPase activity, the relationship of SR Ca^{2+}-ATPase activity to temperature has also been studied. Further, the efficiency of SR Ca^{2+} transport at different temperatures has been evaluated based on the coupling ratio of rate of SR Ca^{2+} uptake and SR Ca^{2+}-ATPase activity.

MATERIALS AND METHODS

All experimental procedures reported herein received prior approval by the University of Alberta Animal Use Committee following the guidelines of the Canadian Council on Animal Care.

Animals. Richardson's ground squirrels (*Spermophilus richardsonii*) were trapped in farm fields near Edmonton, Alberta, Canada. The animals were maintained individually with food and water ad libitum at 22°C, and a 12:12 h light-dark photoperiod. The rectal temperature of the active individuals was typically around 37.5°C. Male Sprague-Dawley rats were obtained from the Biological Sciences Laboratory Animal Services on campus and kept under the same conditions as the ground squirrels.

Isolation of cardiac SR. Crude SR vesicles were isolated by differential centrifugation as described previously (Belke et al. 1987; Belke et al. 1991) with some modifications. Both species were killed by decapitation. Hearts from four rats or ground squirrels were quickly excised and immersed in ice-cold 0.9% NaCl. All subsequent procedures were done below 4°C. After aorta and atria were removed; the ventricles were cut open and blotted. The ventricular tissue was then homogenized in 20 volumes (v/w) of ice-cold isolation solution which contained (in mM): 300 sucrose, 5.0 NaN_3, 0.5 dithiothreitol, 20 3-[N-Morpholino]-propanesulfonic acid (MOPS), pH=7.00. This was performed with a Virtis homogenizer at speed setting 60 for 20 seconds, rest for 10 seconds and then at speed setting 90 for 30 seconds. The crude homogenate was centrifuged at 5000 r/min in a Beckman JA-20 rotor (3020 x g_{max}) for 20 minutes. The supernatant was filtered through 8 ply gauze sponges and centrifuged at 10500 r/min in the same rotor (13300 x g_{max}) for 20 minutes. The pellet was discarded while the supernatant was centrifuged at 33000 r/min in a Beckman TI-70 rotor (110000 x g_{max}) for 35 minutes. The resulting pellet was suspended in a wash solution which contained (in mM): 300 sucrose, 5.0 NaN_3, 0.5 dithiothreitol, 600 KCl, 20 MOPS, pH=7.00, and centrifuged again at 33000 r/min in the TI-70 rotor for 35 minutes. The pellet was resuspended in a storage solution which contained (in mM): 150 KCl, 5.0 NaN_3, 20 MOPS, pH=7.00. The SR vesicles were frozen in liquid nitrogen, stored below -60°C and used for measurement within 3 days. The protein concentration in the SR vesicles was determined by the Bradford (1976) method with bovine serum albumin as the standard.

Measurement of Ca^{2+} uptake. The oxalate-supported Ca^{2+} uptake by SR vesicles at 37, 25, 15 and 5°C was measured by the decrease of optical absorbance of Arsenazo-III at each temperature. The changes of absorbance at a wavelength pair of 654 and 700 nm were monitored in an SLM-AMINCO DW2000 dual-wavelength spectrophotometer equipped with thermostatical chambers. The final reaction medium with a total volume of 2.3 ml contained (in mM): 100 KCl, 20 MOPS, 10 NaN_3, 10 potassium oxalate, 5.0 $MgCl_2$, 5.0 ATP, 0.1 added $CaCl_2$, 15 μM Arsenazo-III, 10 μM ruthenium red and 35-100 μg protein/ml of SR vesicles. The Ca^{2+} uptake was initiated by injecting a mixture of ATP, $MgCl_2$ and $CaCl_2$ with a Hamilton syringe to an otherwise complete reaction medium under vigorous stirring. The pH value of both the reaction medium and injected mixture were adjusted to 7.00 at each assay temperature. The absorbance changes induced by Ca^{2+} were calibrated by addition of 230 nmols of Ca^{2+} (46 μl) into 2.3 ml reaction medium.

Measurement of Ca^{2+}-ATPase activity. The activities of SR Ca^{2+}-ATPase at 37, 25, 15 and 5°C were measured spectrophotometrically with the molybdo-vanadate method (Le Cocq and Inesi 1966). The amount of inorganic phosphate cleaved from ATP in the SR reaction mixture was measured after 2-10 minute incubation at each temperature. The reaction mixture contained (in mM): 100 KCl, 20 MOPS, 10 NaN_3, 10 potassium oxalate, 5.0 $MgCl_2$, 5.0 ATP, 0.1 added $CaCl_2$, 10 μM ruthenium red and 30-85 μg protein/ml of SR vesicles, pH=7.00 at all temperatures. After incubation of 1 ml of the reaction mixture at the desired temperature for 10 minutes, the reaction was started by addition of the SR membrane vesicles. The reaction was stopped after 2-10 minutes by addition of 1 ml of cold colorimetric reagent (R-III) which contained 30% (W/V) trichloroacetic acid, 16 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and 4 mM NH_4VO_3. The solution was cooled on ice and then centrifuged at 13300 x g_{max} for 10 minutes to remove protein precipitate. The solution was diluted 10 times with double deionized water and the amount of inorganic phosphate was measured at 350 nm in a PYE-UNICAM SP1800 spectrophotometer by comparing the absorbance against a standard curve of 0.1-3.5 mM KH_2PO_4. The total ATPase activity was measured in the presence of Ca^{2+}, while the activity of Ca^{2+} independent (basal) ATPase was determined under identical conditions, except that 0.1 mM $CaCl_2$ was replaced by 2 mM ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA). The activity of Ca^{2+} dependent ATPase was obtained by subtracting basal ATPase activity from total ATPase activity.

Statistics. The results were presented as means ± SE. Multiple comparisons of Ca^{2+} uptake rate, Ca^{2+}-ATPase activity, and the coupling ratio at different temperature and between the rats and ground squirrels were performed using

ANOVA (SPSS/PC+) on an IBM PC. P values < 0.05 were considered significant.

RESULTS

SR Ca^{2+} uptake. Figure VI.1 shows the rate of Ca^{2+} uptake by SR in both rats and ground squirrels under a pH value of 7.00. A marked difference ($p<0.05$) in the rate of Ca^{2+} uptake was observed at all temperatures examined from 37 to 5°C. The rate of Ca^{2+} uptake in rat SR was 507±37 nmol/min/mg protein at 37°C. In ground squirrel SR, however, the rate of Ca^{2+} uptake at 37°C was 2400±149 nmol/min/mg protein, which was 3.7 times higher than that of rat SR. As temperature decreased from 37°C, the rate of Ca^{2+} uptake decreased markedly ($p<0.05$) in both rats and ground squirrels. At 5°C, the Ca^{2+} uptake by rat SR was almost negligible, being 17±1 nmol/min/mg protein; whereas the activity of Ca^{2+} uptake by ground squirrel SR was maintained at a significantly high level ($p<0.05$), being 100±14 nmol/min/mg protein.

The rate of Ca^{2+} uptake by SR at different temperatures was analyzed using the Arrhenius plot, in which the natural logarithmic values of SR Ca^{2+} uptake are plotted as a function of reciprocal values of absolute temperature. As can be seen in Figure VI.2, the results can be best fitted with a straight line on linear regression ($r^2=0.999$) for rat SR Ca^{2+} uptake from 37 to 15°C. While at 5°C, the Ca^{2+} uptake by rat SR was minimal, so there is a breakpoint in the Arrhenius plot between 15 and 5°C. The calculated Q_{10} values for rat SR Ca^{2+} uptake were 2.3±0.1 from 37 to 15°C and 6.0±1.0 from 15 to 5°C. For the ground squirrels, on the other hand, a straight line can be best fitted by linear regression ($r^2=0.990$) over the entire 37-5°C range of SR Ca^{2+} uptake, and the Q_{10} was 2.7±0.1 from 37 to 5°C.

SR Ca^{2+}-ATPase activity. The activity of SR Ca^{2+}-ATPase was also measured

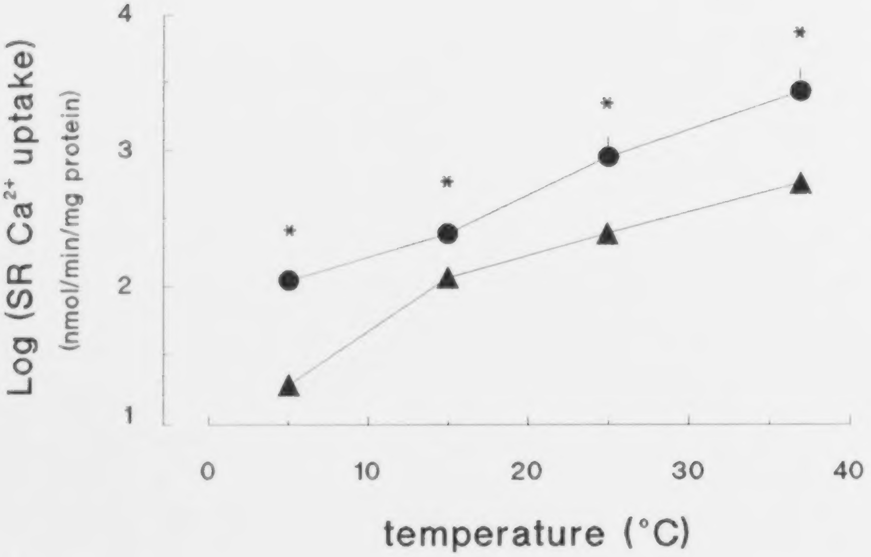

Figure VI.1. Effect of temperature on the rate of cardiac SR Ca^{2+} uptake in rats (triangles) and Richardson's ground squirrels (circles). * significantly different ($p<0.05$) between the rats and ground squirrels at the same temperature. means±SE, n=5-8 at each point.

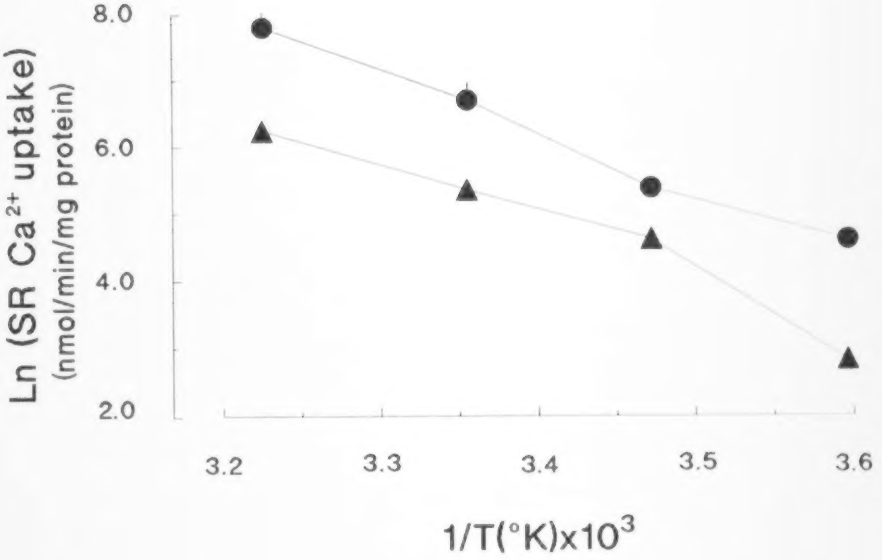

Figure VI.2. The Arrhenius plot of the rate of cardiac SR Ca^{2+} uptake in rats (triangles) and Richardson's ground squirrels (circles). means±SE, n=5-8 at each point.

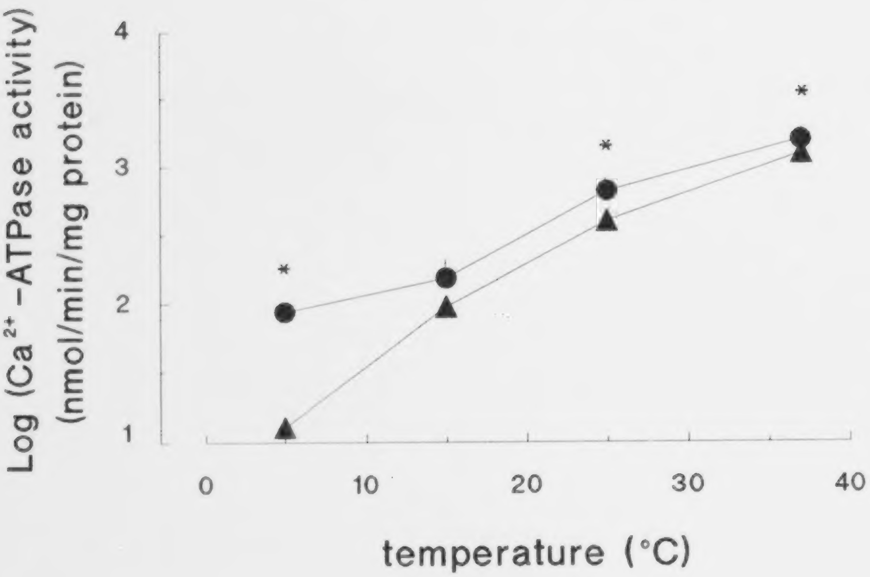

Figure VI.3. Effect of temperature on SR Ca^{2+}-ATPase activity in rats (triangles) and Richardson's ground squirrels (circles). * significantly different ($p<0.05$) between the rats and ground squirrels at the same temperature. means±SE, n=5-8 at each point.

in both rats and ground squirrels (Fig.VI.3). At all temperatures tested between 37 and 5°C, the activities of SR Ca^{2+}-ATPase were always greater in ground squirrels than in rats. However, it was only at 37 and 25°C that the difference was statistically significant ($p<0.05$). The Arrhenius plot of SR Ca^{2+}-ATPase activity in rats showed a curvilinear relationship, whereas a linear relationship ($r^2=0.970$) was seen in ground squirrels (Fig.VI.4). The Q_{10} values for rat SR Ca^{2+}-ATPase activity were 2.6 ± 0.1 between 37 and 25°C, 3.5 ± 0.4 between 25 and 15°C, and 6.8 ± 2.8 between 15 and 5°C, respectively. In ground squirrels, the Q_{10} value was 2.8 ± 0.4 between 37 and 5°C.

Coupling ratio of SR Ca^{2+}-ATPase. The efficiency of Ca^{2+} transport by SR Ca^{2+}-ATPase at different temperatures was evaluated based on the coupling ratio of Ca^{2+} uptake versus Ca^{2+}-ATPase activity (Table VI.1). In rat SR, the coupling ratio was about 0.5-0.7 between 37 and 25°C. At lower temperatures, the coupling ratio increased to about 1.3-1.6 because of the greater inhibitory effect of low temperature on Ca^{2+}-ATPase activity than on Ca^{2+} uptake. In ground squirrels, on the other hand, the coupling ratio did not change significantly ($p>0.05$), being 1.2-1.8 at all temperatures (Table VI.1).

DISCUSSION

Since the yield of crude SR vesicles does not exceed 2 mg per heart in either rats or ground squirrels, the crude SR has been used without further purification in the present study. Although there is some contamination from mitochondrial and sarcolemmal membrane fractions after differential centrifugation, the SR Ca^{2+} uptake and Ca^{2+}-ATPase activity at different temperatures can still be studied because: a) mitochondrial uniporter which transports Ca^{2+} into mitochondria can be inhibited by ruthenium red noncompetitively (Rossi et al. 1973). The mitochondrial Ca^{2+} sequestration can also be inhibited by sodium azide indirectly by interfering with the generation of the proton-motive force across intramitochondrial membrane (Panagia et al. 1985); and b) Ca^{2+} uptake

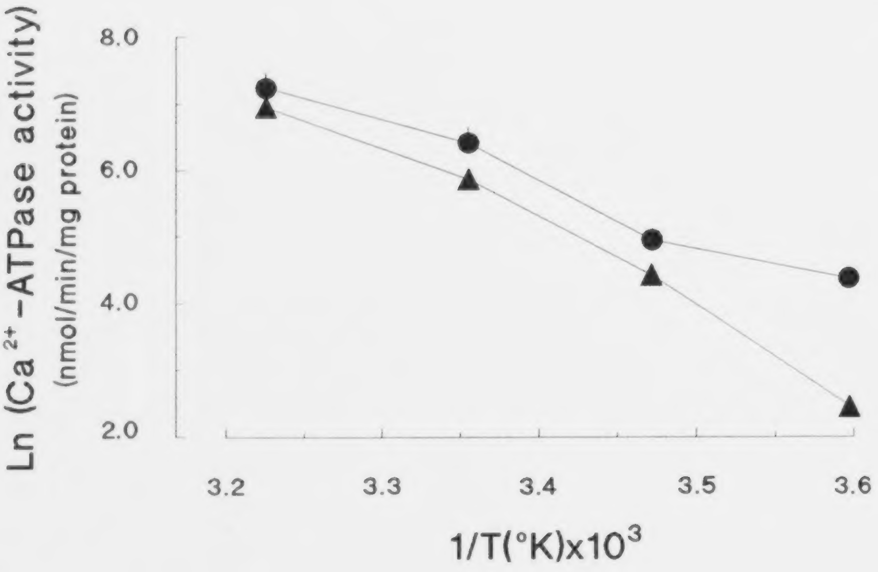

Figure VI.4. The Arrhenius plot of SR Ca^{2+}-ATPase activity in rats (triangles) and Richardson's ground squirrels (circles). means±SE, n=5-8 at each point.

Table VI.1. Rate of Ca^{2+} uptake and activity of Ca^{2+}-ATPase in isolated sarcoplasmic reticulum from rats and Richardson's ground squirrels (G.S.), pH=7.00.

	Temp. (°C)	Ca^{2+} uptake (nmol/min/mg)	Ca^{2+}-ATPase (nmol/min/mg)	Coupling ratio
Rat	37	507±37^{*+}	1040±47^{*+}	0.53±0.07
	25	209±18^{*+}	348±20^{*+}	0.63±0.07
	15	102±14^{*+}	83±24*	1.55±0.40*
	5	17±0.9^{*+}	11±4^{*+}	1.32±0.23*
G.S.	37	2400±149*	1389±150*	1.80±0.14
	25	801±43*	599±41*	1.35±0.05
	15	218±30*	139±14	1.54±0.23
	5	100±14*	79±29	1.18±0.28

* significantly different ($p<0.05$) among the values at different temperatures in either rats or ground squirrels. $^{+}$ significantly different ($p<0.05$) between the rats and ground squirrels at the same temperature. n=5-8 at each.

by SR, but not by sarcolemmal membrane, is greatly facilitated oxalate (Levitsky 1984), so that the disturbance of sarcolemmal membrane fraction on SR Ca^{2+} uptake is minimal when oxalate is included in reaction medium.

In another study with isolated cardiac SR from Richardson's ground squirrels (Belke et al. 1987), the rate of Ca^{2+} uptake is lower than the present study at all temperatures investigated between 37 and 5°C. There may be three possible reasons for this difference. First, a Ca^{2+} electrode is used in the previous study, while a metallochromic dye, Arsenazo-III, is used in the present study to measure the decrease of $[Ca^{2+}]$ in the medium. The ability to detect $[Ca^{2+}]$ changes is much faster in optical measurement with Arsenazo-III. Second, the pH value of the reaction medium is set at 7.385 in the previous study rather than 7.00 as in the present study. It has been demonstrated that an increase in pH value decreases the rate of SR Ca^{2+} uptake (Meltzer and Berman 1984). Third and perhaps the most importantly, ruthenium red which also inhibits Ca^{2+} release from SR is included in the present study but not in the previous study. It has been proposed that a parallel leak of Ca^{2+} from SR may mask the actual rate of SR Ca^{2+} uptake (Feher and Davis 1991). Therefore, when this SR Ca^{2+} leak is inhibited by ruthenium red, particularly at 37°C, the measured net SR Ca^{2+} uptake should be markedly improved.

The present study demonstrates that the rate of Ca^{2+} uptake by isolated cardiac SR is significantly higher in a hibernating species (the Richardson's ground squirrel) than in a non-hibernating species (the rat). Although the temperature sensitivity is somewhat higher in ground squirrels than in rats (Q_{10}=2.7±0.1 versus 2.3±0.1) between 37 and 15°C, the significantly higher absolute value of Ca^{2+} uptake may more than compensate for the greater temperature sensitivity. Thus, at 15°C, the rate of SR Ca^{2+} uptake in ground squirrels is twice that in the rats. At 5°C, the SR Ca^{2+} uptake is minimal in rats, whereas it is maintained at a relatively high level in ground squirrels. The linear relationship in the Arrhenius plots on both SR Ca^{2+} uptake and Ca^{2+}-ATPase activity also suggests that the activation energy for SR Ca^{2+} transport in ground squirrels does not change between 37 and 5°C. In rats, on the other hand, the curvilinear Arrhenius relationship of SR Ca^{2+}-ATPase activity is indicative of a continuous increase of activation energy for SR Ca^{2+}-transport as temperature decreases.

In a previous study, a significantly higher number of Ca^{2+}-release channel/ryanodine-binding receptors has been found in cardiac SR of the Richardson's ground squirrel as compared to that of the sheep (Milner et al. 1991). It is reasonable to assume that the significantly high rate of Ca^{2+} uptake by SR observed in ground squirrels is due to a high density of Ca^{2+}-ATPase in the SR membrane. This is indeed true as we compare the activity of Ca^{2+}-ATPase between rats and ground squirrels (Fig.VI.3 and Table VI.1). Additionally, more efficient Ca^{2+} transport by the Ca^{2+}-ATPase may also contribute to the higher rate of SR Ca^{2+} uptake in the ground squirrels. This can be seen from the greater coupling ratio of Ca^{2+} uptake versus Ca^{2+}-ATPase activity in ground squirrels (Table VI.1). The coupling ratio is 1.80 at 37°C and does not change significantly at lower temperatures. In rats, on the other hand, the coupling ratio is only 0.53-0.63 between 37 and 25°C. At lower temperatures, the coupling ratio increases because the Ca^{2+}-ATPase activity is markedly inhibited.

Taken together, the present study indicates that the rate of Ca^{2+} uptake by isolated cardiac SR is significantly higher in ground squirrels than in rats at all assayed temperatures from 37 to 5°C. This marked difference is probably due to a high density of Ca^{2+}-ATPase in the SR membrane of ground squirrels. On the other hand, higher efficiency of Ca^{2+}-ATPase in Ca^{2+} transport, as evidenced by a greater coupling ratio, may also contribute to the higher rate of Ca^{2+} uptake observed in isolated cardiac SR of ground squirrels. Because the rate of SR Ca^{2+} uptake in ground squirrels remains high at low temperature, it provides a high capacity for regulation of $[Ca^{2+}]_i$ and therefore for normal cardiac contractile function at low temperature as observed in hibernating species.

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VII. IMPROVED RAT HEART FUNCTION AFTER LOW Ca^{2+} PERFUSED PRESERVATION AT 5°C FOR 18 HOURS [5]

INTRODUCTION

Since the first reported by Barnard in 1967, heart transplantation has become accepted as the treatment for end-stage heart disease (Burdine et al 1990). However, the process of heart transplantation is hindered because of the limited number of donor hearts and the relatively short period for which hearts can be preserved (Havel et al. 1991). Although recent advances in organ preservation have led to successful clinical storage of liver, kidney and pancreas for 24 to 48 hours and experimental storage for 48 to 72 hours, clinical storage of the heart is limited to 4 to 6 hours and experimental storage limited to up to 24 hours (Belzer and Southard 1988; Swanson et al. 1988; Southard et al. 1990; Stringham et al. 1992). A fundamental difference between preservation of the heart versus other organs is the mandatory maintenance of contractile function which relies on the intracellular Ca^{2+} homeostasis essential for excitation-contraction coupling (Langer 1990; Bers 1991).

Experimental and clinical studies have indicated that low temperature profoundly affects heart function. In most mammals including humans, severe cardiac arrhythmias, particularly ventricular fibrillation, begin to appear when body temperature (T_b) decreases to 29-25°C in accidental or experimental hypothermia (Nordrehaug 1982; Johansson 1985; Solomon et al. 1989). Further decrease in T_b can result in cardiac arrest. Since isolated cardiac muscle from rats and rabbits develops aftercontractions at low temperature (Liu et al. 1990) and a transient inward current across the sarcolemmal membrane is seen below 20°C in rat cardiac cells (Liu et al. 1991a), it has been suggested that disturbance of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) regulation by cooling is one of the main mechanisms leading to cardiac arrhythmia and cell death during and/or after hypothermia. This has been confirmed by direct measurements of $[Ca^{2+}]_i$ using the fluorescent dye, Indo-1, which indicate a significant increase in $[Ca^{2+}]_i$ in isolated rat ventricular myocytes as temperature is decreased from 37°C to either 15 or 5°C (Liu et al. 1991b). Since an abnormal increase of $[Ca^{2+}]_i$ stimulates energy dependent processes leading to cellular energy-depletion (Farber 1981; Tani 1990), and activates cellular proteases and phospholipases leading to increased oxidative stress and membrane damage, respectively (Fuller et al. 1988; Cohen 1989), a reduction of $[Ca^{2+}]_i$ may be a critical factor to consider in prolonging heart preservation.

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[5] A version of this chapter has been submitted for publication: Liu, B, Lopaschuk, G. D. and Wang, L. C. H. (199x) Improved rat heart function after low Ca^{2+} perfused preservation at 5°C for 18 hours. *Cryobiology.*

To evaluate the effect of $[Ca^{2+}]_i$ on long term heart preservation, we have manipulated the $[Ca^{2+}]$ in the perfusate of isolated rat hearts. This has been initiated at 37°C before cooling, continued during cooling, and maintained while the heart remains at 5°C for varying periods. Our present study indicates that by adjusting extracellular Ca^{2+} to 0.5 mM, heart function can be significantly improved following long term (18 hours) preservation at 5°C.

MATERIALS AND METHODS

All experimental procedures reported herein received prior approval by the University of Alberta Animal Use Committee following the guidelines of the Canadian Council on Animal Care.

Male Sprague-Dawley rats (350-550 g) were anaesthetized with sodium pentobarbital (80 mg/kg) and heparinized (2000 u/kg) intraperitoneally. The hearts were rapidly excised and immersed in cold (4°C) Krebs-Henseleit (K-H) solution which contained (in mM): 118 NaCl, 4.7 KCl, 1.6 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.5 Na_2-EDTA, 2.5 $CaCl_2$, 11.0 glucose, and 2.0 Na-pyruvate, gassed with 95% O_2 and 5% CO_2 (pH 7.40 at 37°C). The hearts were cannulated for both Langendorff perfusion and working heart mode as described by Neely et al. (Neely et al. 1967; Lopaschuk et al. 1986). The hearts were initially perfused, in Langendorff mode, with normal K-H solution at 37°C for 15 minutes with an aortic pressure of 60 mm Hg. The hearts were then perfused randomly under one of three experimental protocols.

Group 1-Controls: The hearts were perfused with K-H solution (2.0 mM Ca^{2+}) at 37°C in working heart mode for 60 minutes. The left atrial filling pressure was 11.5 mm Hg and the aortic pressure 60 mm Hg. At the end of the 60 minute perfusion in working heart mode, an 18 gauge needle was inserted into the left ventricle through the apex of the heart. Left ventricular pressure was measured with a pressure transducer (Gould P23 ID) and recorded on a polygraph (Grass 7E). The signal of pressure development was also stored in an IBM/PC with CODAS, a data acquisition program (DATAQ Instruments Inc, Akron, Ohio) at a sampling rate of 1 kHz for later analysis.

Group 2-Hearts perfused with K-H solution (2.0 mM Ca^{2+}) at 5°C for 1, 6 or 18 hours: The temperature was gradually decreased to 5°C during a 60 minute period while the hearts were perfused in Langendorff mode. After the hearts had been continuously perfused at 5°C for 1, 6 or 18 hours, the temperature was raised over a 40 minute period to 37°C. The hearts were then perfused in Langendorff mode for 10 minutes, followed by perfusion in working heart mode for 60 minutes. The left ventricular pressure was measured as described in group 1 at the end of the 60 minute perfusion in working heart mode.

Group 3-Hearts perfused at 5°C for 18 hours in K-H solution with different Ca^{2+} concentrations: The hearts were perfused at 37°C in Langendorff mode for 30 minutes in a modified K-H solution with different Ca^{2+} concentrations (0.7, 0.5, or 0.35 mM). The temperature was then decreased to 5°C over a 60 minute period. After the hearts had been continuously perfused in Langendorff mode at this temperature for 18 hours in the same modified K-H solution, the temperature was raised to 37°C over a 40 minute period. After 5 minutes at 37°C, the perfusate was switched to K-H solution (2.0 mM Ca^{2+}) for 5 minutes in Langendorff mode, and then in working heart mode for 60 minutes. The left ventricular pressure was then measured as described in Group 1.

Coronary flow (CF) at 37 and 5°C was measured by collecting the effluent in graduated cylinders as hearts were perfused in Langendorff mode. At the end of the experiments, the hearts were cut from the cannulae and the ventricles cut open and blotted on filter paper. The dry weight of heart tissue was obtained by drying the tissue for 48 hours in an oven at 80°C. The CF was expressed as milliliters per gram of dry weight of the heart tissue. The heart rate (HR), left ventricular systolic pressure (LVSP), and the maximal rate of pressure development ($+dP/dt_{max}$) were measured with a data analysis program, POSTACQ (DATAQ Instruments Inc. Akron, Ohio).

All solutions were made from double de-ionized distilled water, and filtered through 0.8 μm Millipore filters before use. As the temperature was lowered from 37 to 5°C, the pH of the oxygenated K-H solutions decreased from 7.40 to 7.25; however, no effort was made to correct the pH value at 5°C.

Results are expressed as means ± SE. Multiple comparisons were made by one way analysis of variance using student-Newman-Keuls posthoc analysis performed with a statistical program, SPSS/PC+(ANOVA). P values < 0.05 were considered significant.

RESULTS

Effect of hypothermic duration on recovery of heart function. Figure VII.1 shows the changes in cardiac function after the hearts were perfused at 5°C for different periods in K-H solution with 2.0 mM free Ca^{2+}. As the control after hearts were perfused at 37°C for 60 minutes without cooling in working heart mode (n=6), the HR was 217±4 beats/min, LVSP was 118±5 mm Hg, and $+dP/dt_{max}$ was 2793±109 mm Hg/s. Following rewarming from perfusion at 5°C for 1 hour, 6, or 18 hours (n=6 in each group), the HR recovered completely. The LVSP and $+dP/dt_{max}$ also recovered after 1 hour perfusion at 5°C. After 6 hour perfusion at 5°C, the LVSP and $+dP/dt_{max}$ were lower than the control, but not statistically significant. However, the product of HR times LVSP was significantly lower than that in the control and 1-hour preservation groups. Following 18 hours at 5°C, the LVSP, $+dP/dt_{max}$, and the product of HR times LVSP were significantly ($p<0.05$) reduced. The ventricular diastolic pressure after 18 hour perfusion at 5°C increased markedly from 0 mm Hg to 10±2 mm Hg, indicating an incomplete relaxation probably due to increased $[Ca^{2+}]_i$.

Recovery of cardiac function after 18 hour perfusion at 5°C in low Ca^{2+} solutions. Free Ca^{2+} concentration in the K-H solution was varied from 2.0 mM to 0.7, 0.5, or 0.35 mM during the initial 30 minute perfusion at 37°C and during the 18 hour cold perfusion at 5°C (n=6 in each group). The recovery of heart function after rewarming is shown in Figure VII.2. When Ca^{2+} was decreased from 2.0 mM to 0.7 and 0.5 mM, no significant changes in HR were observed, however, the LVSP and $+dP/dt_{max}$ after rewarming were significantly higher than that in 2.0 mM Ca^{2+}-group. The recovery of both LVSP and $+dP/dt_{max}$ was better in 0.5 mM Ca^{2+}-group than that in 0.7 mM Ca^{2+}-group. On the other hand, no significant changes in diastolic pressure in either 0.7 or 0.5 mM Ca^{2+}-group were observed as compared with the control value. When Ca^{2+} concentration was further decreased to 0.35 mM, reduced HR, LVSP, and $+dP/dt_{max}$ were seen compared to hearts perfused with 0.5 mM Ca^{2+}.

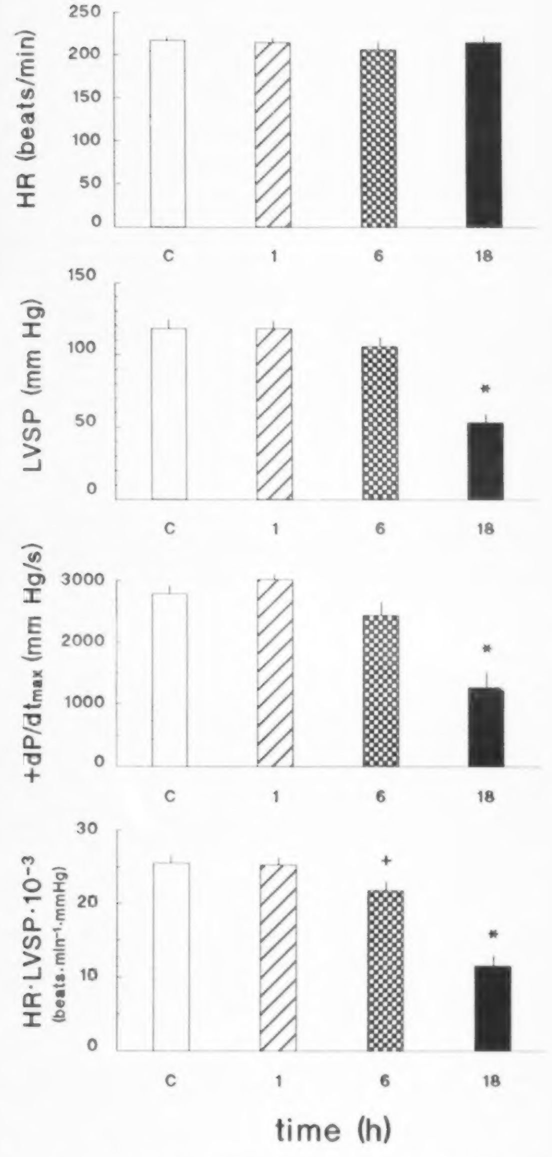

Figure VII.1. Heart rate (HR), left ventricular systolic pressure (LVSP), the maximal rate of pressure development ($+dP/dt_{max}$), and the product of HR times LVSP (HR·LVSP) at 37°C after hearts were perfused with 2.0 mM Ca^{2+} at 5°C for 1, 6, or 18 hours. Control hearts (C) were perfused at 37°C for 1 hour without cooling. * significantly ($p<0.05$) lower than any other groups, + significantly ($p<0.05$) lower than the control. means+SE, n=6 in each group.

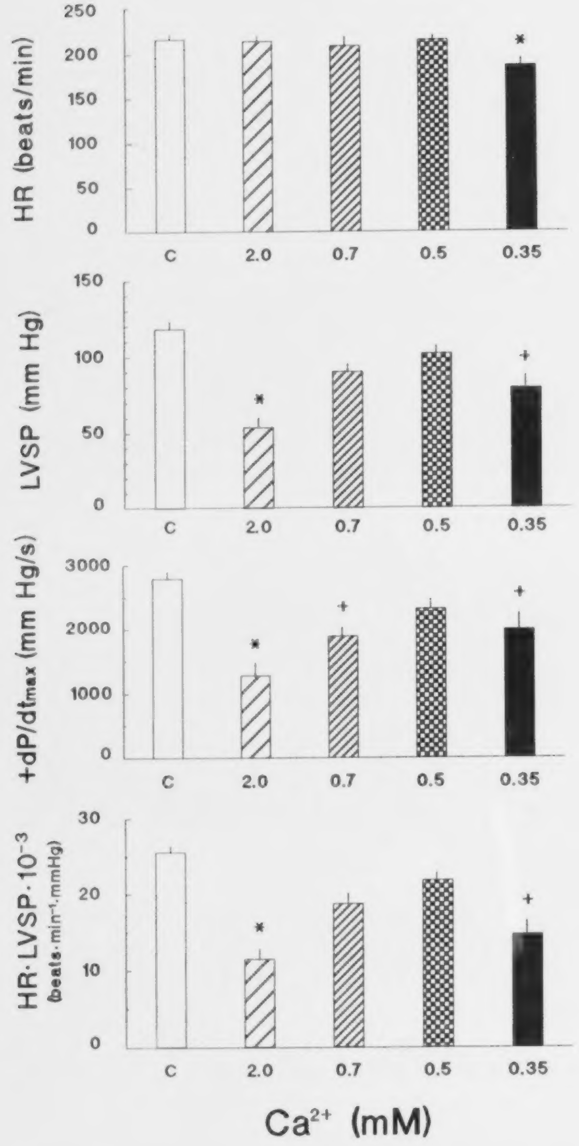

Figure VII.2. Heart rate (HR), left ventricular systolic pressure (LVSP), the maximal rate of pressure development (+dP/dt$_{max}$), and the product of HR times LVSP (HR·LVSP) at 37°C in 2.0 mM Ca^{2+} solution after hearts were perfused with varied [Ca^{2+}] at 5°C for 18 hours. C, control group. * significantly ($p<0.05$) lower than any other groups, + significantly ($p<0.05$) lower than the control. means+SE, n=6 in each group.

Coronary flow following myocardial cooling. In hearts perfused with 2.0 mM free Ca^{2+} at 5°C for 1 or 6 hours, CF during rewarming recovered completely to the control value of 54.0±3.0 ml/min/g of dry tissue (Fig.VII.3, $n=6$ in each group). When perfusion duration at 5°C was increased to 18 hours, however, the CF after rewarming to 37°C was significantly reduced, 40.2±2.6 ml/min/g of dry tissue ($n=6$).

The CF in hearts cooled and rewarmed with different Ca^{2+} is shown in Table VII.1. At 37°C, CF was highest in hearts perfused with 0.7 mM Ca^{2+}, lower in hearts perfused with 0.5 and 2.0 mM Ca^{2+} and the lowest in hearts perfused with 0.35 mM Ca^{2+}. When the temperature was lowered to 5°C, CF in all Ca^{2+} solutions decreased significantly and continued to decrease as perfusion duration at 5°C lengthened. After rewarming to 37°C in K-H solution containing 2.0 mM free Ca^{2+}, CF increased to 49.4±1.0 and 48.1±4 ml/min/g dry tissue in the 0.7 and 0.5 mM Ca^{2+}-groups, respectively; these values were lower than, but not significantly different from the control group (Fig.VII.4). On the other hand, the values of CF in 2.0 and 0.35 mM Ca^{2+}-groups were significantly lower than the control and other two groups.

DISCUSSION

Two different responses of heart to cold have been observed in mammals (Wang 1988; Johansson 1991). Animals which can hibernate in a cold environment, such as Richardson's ground squirrels, maintain cardiac contractile function at 5°C even though HR is greatly reduced (2-10 beats/min at 5°C; Wang 1988). On the other hand, the hearts of humans and commonly used laboratory animals, such as rats, guinea pigs and dogs, develop severe cardiac arrhythmias when cooled and stop beating when temperature is lowered below 16-10°C depending on the species (Nordrehaug 1982; Johansson 1985; Solomon et al. 1989). Studies have indicated that precise regulation of $[Ca^{2+}]_i$ is critical in maintaining cardiac function at low temperature. For instance, direct

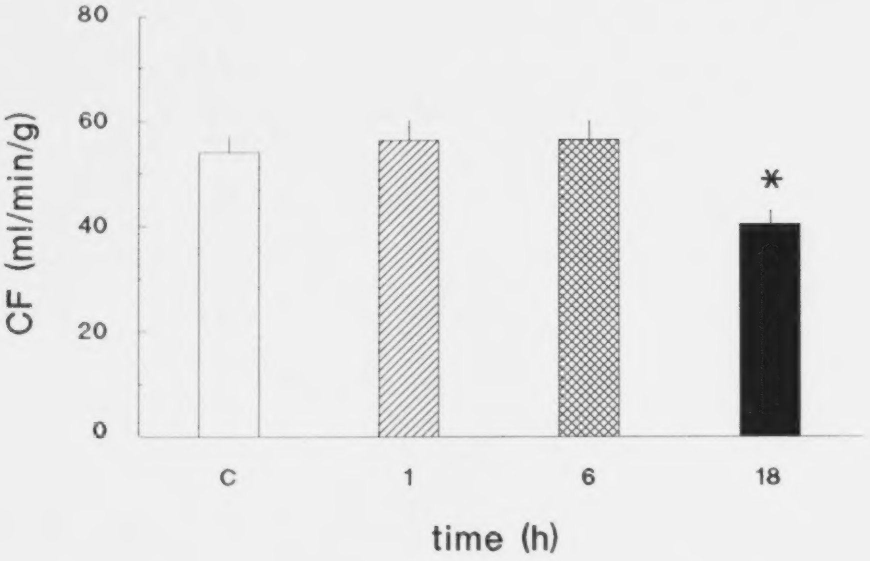

Figure VII.3. Coronary flow (CF, ml/min/g of dry weight) at 37°C after hearts were perfused at 5°C with 2.0 mM Ca^{2+} for 1, 6, or 18 hours. Control group (C) was perfused at 37°C for 1 hour without cooling. * significantly ($p<0.05$) lower than any other groups. means+SE, n=6 in each group.

Table VII.1. Coronary flow (ml/min/g of dry weight) in different Ca^{2+} solutions at 37 and 5°C.

	A	B*	C	D	E+	F
Ca^{2+}	37°C	5°C	5°C	5°C	5°C	37°C[1]
(mM)	(15 min)	(0.5 h)	(1 h)	(6 h)	(18 h)	(5 min)
2.0	56.5±3.6	47.2±1.8	44.4±6.1	33.9±1.7	30.1±1.9	40.2±2.6
0.7	71.3±2.3	28.7±5.5	n.d.	n.d.	26.9±5.7	41.3±9.4
0.5	61.5±4.9	38.7±4.7	n.d.	n.d.	26.7±1.2	45.9±4.4
0.35	54.3±3.3	42.2±1.4	n.d.	n.d.	24.8±6.2	35.2±4.7

1. After rewarming.

* Significantly ($p<0.05$) different between A and B in each Ca^{2+} solution.

+ Significantly ($p<0.05$) different between B and E in each Ca^{2+} solution.

n.d. Not determined.

n=6 in each group.

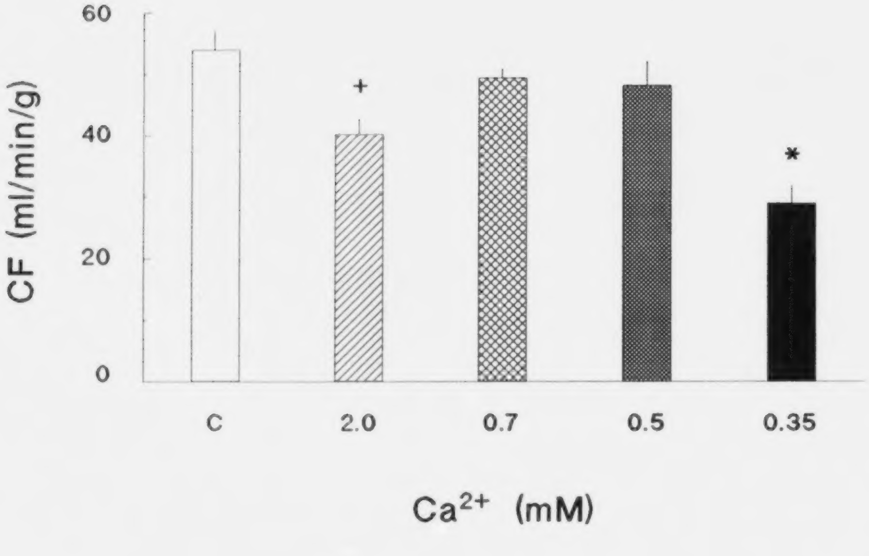

Figure VII.4. Coronary flow (CF, ml/min/g of dry weight) after hearts were perfused with different Ca^{2+} at 5°C for 18 hours and rewarmed to 37°C in 2.0 mM Ca^{2+}. C, control group. * significantly ($p<0.05$) lower than any other groups, + significantly ($p<0.05$) lower than group C and groups with 0.7 and 0.5 mM Ca^{2+}. means+SE, n=6 in each group.

measurement of $[Ca^{2+}]_i$ with the fluorescent dye Indo-1 has shown that the $[Ca^{2+}]_i$ in rat ventricular myocytes increases significantly from 139 nM at 37°C to 300 nM at 5°C (Liu et al. 1991b). Paralleling this change in $[Ca^{2+}]_i$ is an increase in spontaneous contraction of the myocytes from less than 10% at 37°C to nearly 100% at 5°C, suggesting disturbed cardiac function at 5°C due to $[Ca^{2+}]_i$ overload (Liu et al. 1991b). On the other hand, the cardiac $[Ca^{2+}]_i$ in a hibernating species, the Richardson's ground squirrel, increases from 103 nM at 37°C to only 168 nM at 5°C (Liu and Wang 1993), which is significantly less than that seen in non-hibernating species. It is therefore apparent that $[Ca^{2+}]_i$ overload is a critical problem in the maintenance of cardiac function at low temperature. As a result, a remedial measure which reduces $[Ca^{2+}]_i$ overload should be beneficial in prolonging of hypothermic heart preservation.

Since $[Ca^{2+}]_i$ is greatly influenced by changes in the extracellular $[Ca^{2+}]$, a reduction in $[Ca^{2+}]$ in the perfusion solution will reduce intracellular Ca^{2+}. Evidence has shown that reduction of extracellular $[Ca^{2+}]$ reduces the Ca^{2+} influx through the voltage-dependent Ca^{2+} channels in the sarcolemmal membrane (Reuter 1967), Ca^{2+} accumulation in the SR (Fabiato 1985), Ca^{2+} transient in the cell (Marban et al. 1987; Lee et al. 1987), and end-diastolic $[Ca^{2+}]_i$ (Lee et al. 1987). Although many hypothermic ischemic preservation solutions employ zero $[Ca^{2+}]$ to eliminate Ca^{2+} influx across the sarcolemmal membrane, two main problems remain. The first is cell injury due to the Ca^{2+} paradox on reperfusion with normal Ca^{2+} solution or blood (Chapman and Tunstall 1987); the second is the ineffectiveness in preventing Ca^{2+} overload at low temperature probably due to net leaks of Ca^{2+} from intracellular stores as a result of the inhibitory effect of low temperature on Ca^{2+} uptake into SR (Harigaya and Schwartz 1969; Suko 1973, and Chapter VI). It is therefore reasonable to argue that the cellular Ca^{2+} load should be reduced before the heart is preserved at low temperature. To test this hypothesis, we have first perfused isolated rat hearts with low Ca^{2+} K-H solution (0.7 mM to 0.35 mM) at 37°C for 30 minutes in an effort to reduce the

$[Ca^{2+}]_i$ prior to hypothermic preservation. Although the final $[Ca^{2+}]_i$ accomplished by this measure is unknown, previous research (Reuter 1967; Fabiato 1985; Marban et al. 1987; Lee et al. 1987) indicates that this protocol decreases $[Ca^{2+}]_i$. The optimal free $[Ca^{2+}]$ in the solution for rat hearts is 0.5 mM; at higher or lower $[Ca^{2+}]$ a reduction of HR, LVSP, $+dP/dt_{max}$ and CF is evident. These observations are consistent with our hypothesis that by reducing the $[Ca^{2+}]_i$ prior to cooling and during hypothermic preservation, cardiac function can be preserved without deterioration. These results are consistent with other studies (Takahashi et al. 1989; Batty et al. 1989) which show optimal recovery of cardiac output and minimal release of creatine kinase if 0.5-0.6 mM Ca^{2+} is included in St. Thomas' Hospital cardioplegic solution administered to ischemic rat hearts stored at 7.5°C for 6 hours (Takahashi et al. 1989). A recent study on neonatal lambs also indicates that low $[Ca^{2+}]$ during preischemic hypothermia and early reperfusion offsets deleterious effect of hypothermia on recovery of heart function (Aoki et al. 1993). Our results also demonstrate that poor recovery of cardiac function after cold perfusion occurs in the presence of 0.35 mM Ca^{2+}. This may be due to a dysfunction of the smooth muscle cells since CF is greatly reduced after rewarming. It may also be due to the slow HR since function of the pace-maker cells is highly Ca^{2+}-dependent (Hagiwara et al. 1988).

Our present study has shown that functional recovery of isolated rat hearts after hypothermic perfusion depends on two factors: the duration of cold perfusion and the $[Ca^{2+}]$ in the perfusion medium. Under our experimental conditions, the preload and afterload of working hearts are kept constant at 11.5 and 60 mm Hg, respectively. Since the HR does not change significantly except that in the 0.35 mM Ca^{2+}-group, the $+dP/dt_{max}$ is a direct measurement of cardiac contractility (Opie 1991). Hearts can recover completely after 1 hour perfusion at 5°C in K-H solution with 2.0 mM free Ca^{2+}. After 6 hour perfusion at 5°C, the LVSP and $+dP/dt_{max}$ are lower than the control, but not statistically significant. However, the product of HR times LVSP decreases significantly ($p<0.05$). This indicates that the recovery of heart function starts to decrease after 6 hour perfusion at 5°C in 2.0 mM Ca^{2+}. Significant decreases in LVSP, $+dP/dt_{max}$ and CF, and a significant increase in left ventricular diastolic pressure result when the duration of hypothermia is prolonged to 18 hours. As discussed above, this functional deterioration after 18 hours of cold perfusion may be partially or totally prevented by reducing the $[Ca^{2+}]$ in the perfusion medium. Since we have not arrested hearts with cardioplegic solution but rather perfused hearts with varied $[Ca^{2+}]$ continuously when temperature is decreased to 5°C over a 60 min period, the cardiac contraction and energy status may be different, this may be related to functional recovery upon rewarming. The high-energy phosphates in hearts following cold perfusion with different Ca^{2+} at 5°C need to be measured in further studies.

In conclusion, the preservation of cardiac function can be significantly improved in 18 hour hypothermically (5°C) perfused rat hearts when the $[Ca^{2+}]$ in the K-H perfusion medium is reduced from 2.0 to 0.5 mM. Higher or lower $[Ca^{2+}]$ results in poor recovery of cardiac function following rewarming. Our results indicate that reducing $[Ca^{2+}]_i$ prior to and during cold perfusion is a critical factor in successful cardiac preservation. Reduction of $[Ca^{2+}]_i$ by pre-perfusion with low Ca^{2+} (0.5 mM) can yield significant benefits both in prolonging the duration of cardiac preservation and in the recovery of heart function after rewarming.

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VIII. GENERAL DISCUSSION AND CONCLUSIONS

Cardiac contractile responses to low temperature are markedly different between hibernating and non-hibernating mammals. Hibernators' hearts contract smoothly even at 5-0°C, whereas those of non-hibernators' become severely arrhythmic at 30-16°C and stop beating at 16-10°C. Since Ca^{2+} plays an important role in contractile function of the heart, cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) and its regulation at low temperature have been investigated in the present study, in both hibernating and non-hibernating species. Before we come to any conclusions, the $[Ca^{2+}]_i$ homeostasis and cardiac function need to be discussed.

1. $[Ca^{2+}]_i$ Homeostasis and Cardiac Excitation-contraction Coupling.

Heart function, in terms of the contraction-relaxation cycle, is essentially regulated by changes in $[Ca^{2+}]_i$ (Sperelakis 1989; Langer 1990; Bers 1991; Callewaert 1992; Stern and Lakatta 1992). According to the Ca^{2+}-induced Ca^{2+} release hypothesis originally proposed by Endo (1977) in skeletal muscle and later developed by Fabiato (1983) in cardiac muscle, Ca^{2+} influx across the sarcolemma following membrane depolarization triggers the release of a large amount of Ca^{2+} from sarcoplasmic reticulum (SR). The resulting rapid increase in $[Ca^{2+}]_i$ activates the myofilaments and initiates mechanical contraction. The major route for Ca^{2+} influx is through voltage-dependent Ca^{2+} channels; however, recent evidence also suggests that when sarcolemmal membrane is depolarized Ca^{2+} can enter cardiac cells via a Na^+-Ca^{2+} exchange mechanism (Beuckelmann and Wier 1989; Bers et al. 1990). In addition to triggering Ca^{2+} release from the SR, the Ca^{2+} influx may also initiate mechanical contraction directly (Rich et al. 1988). Although charge movement across the sarcolemma during membrane depolarization induces Ca^{2+} release from the SR in skeletal muscle cells, evidence from experiments with skinned muscle fibers (Fabiato 1985a; Fabiato 1986) and more recently studies of photolysis of caged Ca^{2+} in intact cells (Näbauer et al.

1989; Niggli and Lederer 1990), indicates that SR Ca^{2+} release in mammalian cardiac cells is not dependent on changes in membrane voltage, but is triggered directly by an influx of Ca^{2+} rather than other ions such as H^+, K^+ and Na^+. The Ca^{2+} transients during excitation-contraction coupling have been studied extensively with the fluorescent Ca^{2+} indicators (Fura-2 and Indo-1) in isolated ventricular myocytes (Wier et al. 1987; Li et al. 1988; Takamatsu and Wier 1990; Sollott et al. 1992; Spurgeon et al. 1992; Lipp et al. 1992) and with gated ^{19}F nuclear magnetic resonance spectroscopy in the intact hearts (Marban et al. 1988). $[Ca^{2+}]_i$ increases rapidly to a value of 500-1000 nM during the contracting phase. In the resting diastolic state, $[Ca^{2+}]_i$ is maintained at 100-150 nM.

It is well established that precise regulation of $[Ca^{2+}]_i$ is essential to heart function. Following muscle contraction, the Ca^{2+} released from the SR must be sequestered back into the SR lumen and the excess Ca^{2+} that has entered the cell be extruded from the cell. Any disruption of these processes results in an abnormal increase of $[Ca^{2+}]_i$. Studies have indicated that an abnormal increase of $[Ca^{2+}]_i$ triggers spontaneous release of Ca^{2+} from the SR (Lakatta 1992) and induces a transient inward current and afterdepolarizations of sarcolemmal membrane (Berlin et al. 1989) leading to triggered cardiac arrhythmias (Cranefield and Wit 1979). An abnormal increase in $[Ca^{2+}]_i$ also impairs cellular metabolism and membrane integrity. Studies have shown that an increase in $[Ca^{2+}]_i$ accelerates energy-dependent processes in the cell, leading to an energy-depleted state (Tani 1990). Further, an increase in $[Ca^{2+}]_i$ enhances production of free radicals to cause further cell injuries by activating proteases such as calpain (Cohen 1989). In addition, the activation of phospholipases by increased $[Ca^{2+}]_i$ reduces membrane integrity leading to an increase in permeability (Das et al. 1986). All of these changes will in turn cause further increase in $[Ca^{2+}]_i$ leading to a Ca^{2+}-overload state and eventually cell death. In clinical and experimental observation, an abnormal increase in cardiac $[Ca^{2+}]_i$ has been characteristic of the end stage of heart failure in humans (Gwathmey et al. 1987;

Morgan et al. 1990; Beuckelmann and Erdmann 1992), in ischemia and reperfusion-injured hearts in various animals (Allen and Orchard 1987; Steenbergen et al. 1987; Nayler et al. 1988; Tani 1990).

2. Mechanisms Responsible for $[Ca^{2+}]_i$ Regulation.

Maintenance of $[Ca^{2+}]_i$ at low levels during resting diastolic state involves: 1) Ca^{2+} uptake by SR due to the pumping activity of SR Ca^{2+}-ATPase; 2) Ca^{2+} extrusion out of the cell via Na^+-Ca^{2+} exchanger and Ca^{2+}-ATPase in the sarcolemmal membrane; 3) Ca^{2+} uptake by mitochondria via a Ca^{2+} uniporter; and 4) Ca^{2+} binding to cytosolic soluble molecules. Studies have shown that the contribution to the $[Ca^{2+}]_i$ regulation is different among these mechanisms. SR Ca^{2+}-ATPase is essentially responsible for Ca^{2+} uptake into the SR lumen (Tada et al. 1989). Its activity is regulated by the protein phospholamban. The affinity of SR Ca^{2+}-ATPase to Ca^{2+}, in terms of K_m value, is typically 1-5 μM. When phospholamban is phosphorylated, however, the K_m can decrease 3-5 fold to about 0.2 μM (Shamoo et al. 1985). The rate of SR Ca^{2+} uptake is typically 100-2500 nmol/min/mg protein in isolated cardiac SR from various mammals in *in vitro* studies depending on different species and experimental conditions (Chamberlain et al. 1983; Feher et al. 1988; Feher and Davis 1991).

In contrast to SR Ca^{2+}-ATPase, the activity of sarcolemmal Ca^{2+}-ATPase is regulated by calmodulin. For instance, the K_m for Ca^{2+} is ~11 μM and the maximal rate (V_{max}) for Ca^{2+} transport is ~10 nmol/min/mg protein in sarcolemmal membrane isolated from dog hearts (Caroni and Carafoli 1981a,b). After addition of calmodulin, the K_m decreases to ~0.3 μM while the V_{max} increases to ~31 nmol/min/mg protein. On the other hand, the K_m and V_{max} for the Na^+-Ca^{2+} exchanger in sarcolemmal membrane are typically 0.6-1.0 μM (Miura and Kimura 1989), and 1500 nmol/min/mg protein, respectively (Reeves and Philipson 1989). The Na^+-Ca^{2+} exchanger can transport Ca^{2+} either inward or outward depending on intracellular Na^+ and $[Ca^{2+}]_i$, and membrane potential

(Noble 1984; Kimura et al. 1986; Mechmann and Pott 1986; Ravens and Wettwer 1989; Allen et al. 1989; Beuckelmann and Wier 1989). A calmodulin binding domain and a potential phosphorylation site have recently been identified in the cytoplasmic domain of the Na^+-Ca^{2+} exchanger, which indicates that the activity of the Na^+-Ca^{2+} exchanger is also regulated by calmodulin and cAMP-dependent protein kinase (Nicoll et al. 1990). Since the Ca^{2+}-ATPase in sarcolemmal membrane shows a high affinity (low K_m) but a low capacity (low V_{max}) for Ca^{2+} transport as compared with the Na^+-Ca^{2+} exchanger, it has been proposed that the Na^+-Ca^{2+} exchanger is the major mechanism for extrusion of Ca^{2+} which enters the cell via the voltage-dependent Ca^{2+} channels during membrane excitation.

Ca^{2+} uptake by the mitochondria is carried out by a uniport system down a large electrochemical gradient created by proton extrusion, a process which is linked to the pathway of electrons in the respiratory chain (Crompton 1985). *In vitro* studies have shown that the K_m value for mitochondrial Ca^{2+} sequestration is about 10 μM (Carafoli 1987). This suggests that Ca^{2+} uptake by mitochondria is essentially for its own function, i.e. activation of intramitochondrial dehydrogenases (McCormack and Denton 1989), and that mitochondria do not play an important role in regulating the beat-to-beat change of $[Ca^{2+}]_i$ under physiological conditions (Carafoli 1987; Bers and Bridge 1989; Hansford 1991; Hansford 1992).

In addition to the above mechanisms, cytosolic soluble molecules may also provide a buffer system for the regulation of $[Ca^{2+}]_i$. For instance, a cytosolic Ca^{2+} binding protein parvalbumin has been found in skeletal muscle cells (Gillis et al. 1982). With the limited information available regarding cardiac cells, one may speculate that the binding of Ca^{2+} to cytosolic molecules such as calmodulin, myosin and troponin is essentially for their cellular functions in the processing of the signal rather than to buffer $[Ca^{2+}]_i$ (Robertson et al. 1981; Carafoli 1987).

In a recent study on intact cardiac cells, competition for Ca^{2+} transport during relaxation has been compared among the Ca^{2+} uptake by SR, Ca^{2+} extrusion by either the Na^+-Ca^{2+} exchanger or Ca^{2+}-ATPase in sarcolemmal membrane, and Ca^{2+} uptake by mitochondria (Bers et al. 1992). The Ca^{2+} uptake by SR is 3-4 times faster than by Na^+-Ca^{2+} exchange, while mitochondrial Ca^{2+} uptake and sarcolemmal Ca^{2+}-pumping action are ~35-50 times slower than the Na^+-Ca^{2+} exchange. This indicates that SR Ca^{2+} uptake and sarcolemmal Na^+-Ca^{2+} exchange are the primary mechanisms responsible for reducing $[Ca^{2+}]_i$ during cardiac relaxation and maintaining $[Ca^{2+}]_i$ at low levels.

3. Evidence of Ca^{2+}-overload at Low Temperature in Cardiac Cells of Non-hibernating Mammals.

Voltage-clamp experiments have shown that a transient inward current can be recorded in rat ventricular cells at 20°C (Liu et al. 1991a). The magnitude of this transient inward current is even more pronounced when temperature is further decreased. When mechanical contraction of rat and rabbit ventricular muscles is examined, arrhythmic aftercontractions are observed below 15°C, accompanied by a marked increase in resting tension (Liu et al. 1990). Since these electrical and mechanical changes are generally the consequence of an abnormal increase of $[Ca^{2+}]_i$, it has been proposed that low temperature has a marked inhibitory influence on the mechanisms responsible for regulating $[Ca^{2+}]_i$. The present study provides direct evidence that $[Ca^{2+}]_i$ increases significantly at low temperature in isolated ventricular myocytes from the non-hibernating species, the rat, whereas the increase of $[Ca^{2+}]_i$ is much less in the hibernating species, the Richardson's ground squirrel (Chapters I & II; Liu et al. 1991b; Liu and Wang 1993).

4. Temperature Sensitivity of Various Mechanisms Responsible for the Regulation of $[Ca^{2+}]_i$.

Since impaired $[Ca^{2+}]_i$ homeostasis in cardiac cells at low temperature has been observed in non-hibernating species, it is of interest to compare the temperature sensitivity, in terms of Q_{10} value, of the different mechanisms for $[Ca^{2+}]_i$ regulation. Q_{10} is defined as the ratio of physiological reaction rates when the temperature difference is 10°C. It may be possible through such a comparison that the most temperature-sensitive process may be identified as the limiting factor in maintaining $[Ca^{2+}]_i$ in non-hibernating species.

Ca^{2+} influx through the voltage-dependent Ca^{2+} channels is a major extracellular source to increase $[Ca^{2+}]_i$. Electrophysiological studies have shown that the Q_{10} values of the peak Ca^{2+} current (I_{Ca}) between 37 and 20°C are 2.08-2.96 in guinea pig (Cavalie et al. 1985; Herve et al. 1992), and ~3.2 in rat ventricular myocytes (Mitchell et al. 1983, Liu et al. 1991a). As for hibernating species, the magnitude of I_{Ca} is not influenced by low temperature significantly in the European hedgehog, i.e. Q_{10} ~1.0 (Liu et al. 1991a), while that of the Richardson's ground squirrel decreases with temperature, Q_{10} being 1.92 (Herve et al. 1992).

The Q_{10} value for the peak Na^+-Ca^{2+} exchange current measured with patch-clamp techniques is 3.6-4.0 in rat and guinea pig ventricular myocytes when evaluated between 35 and 25°C or 21°C (Kimura et al. 1987, Niggli and Lederer 1991; Lederer et al. 1992). In sarcolemmal membrane vesicles isolated from rabbit heart, the Ca^{2+} affinity of the Na^+-Ca^{2+} exchanger does not change as temperature decreases from 37 to 7°C, while the velocity of Na^+-Ca^{2+} exchange decreases with a Q_{10} of 2.4 between 37 and 27°C, and 6.1 between 22 and 12°C, respectively. The Arrhenius plot of the velocity of Na^+-Ca^{2+} exchange clearly shows a breakpoint around 22°C and activation energy increases markedly below this temperature (Bersohn et al. 1991). Similar results are observed in isolated sarcolemmal vesicles from dog heart, in which the Q_{10} of the Na^+-Ca^{2+} exchange velocity is 2.0 between 37 and 21°C, and 5.5 between 21 and 7°C, respectively (Tibbits et al. 1992). In poikilotherms, on the other hand, the activity of Na^+-

Ca^{2+} exchange is less influenced by low temperature. In isolated sarcolemmal vesicles of trout heart, the Q_{10} of Na^+-Ca^{2+} exchange velocity is only 1.2 over the temperature range from 37 to 7°C (Tibbits et al. 1992). In a similar membrane preparation of frog heart, the Q_{10} value is ~1.4 and ~2.1 at 37-22°C and 22-10°C, respectively (Bersohn et al. 1991). To date there is no information available on the Na^+-Ca^{2+} exchange activity in mammalian hibernators.

The rate of Ca^{2+} uptake by SR is also decreased at low temperature. In isolated SR vesicles from dog and rabbit heart, the Q_{10} is ~2.8 between 35 and 10°C and a linear relationship has been observed in the Arrhenius plot of both Ca^{2+} uptake rate and Ca^{2+}-ATPase activity (Suko 1973). In the present study, the Q_{10} value of rat SR Ca^{2+} uptake is 2.3 between 37 and 15°C and increases significantly to 6.0 between 15 and 5°C. On the other hand, the Q_{10} is 2.6 between 37 and 5°C in hibernating species, the Richardson's ground squirrels (Chapter 6).

When these observations are considered together, the activity of the Na^+-Ca^{2+} exchange in sarcolemmal membrane seems to be the most temperature-sensitive in non-hibernating species. Assuming that a balance between Ca^{2+} influx via the Ca^{2+} channels and Ca^{2+} extrusion via Na^+-Ca^{2+} exchange is achieved at 37°C, one may expect a net Ca^{2+} gain at low temperature because of the differential temperature sensitivity of these mechanisms. In this case, a gradual increase in $[Ca^{2+}]_i$ will increase Ca^{2+} loading in the SR, and therefore the magnitude of muscle contraction (more discussions below). However, SR Ca^{2+} loading will be saturated as $[Ca^{2+}]_i$ increases continuously at low temperature. The resulting abnormal increase in $[Ca^{2+}]_i$ will eventually trigger spontaneous Ca^{2+} release from the SR and induce severe cardiac arrhythmias. These have indeed been observed in cardiac cells of non-hibernating species at low temperature.

In mammalian hibernators, the temperature sensitivity of SR Ca^{2+} uptake is similar to that of non-hibernators. However, the inhibitory effect of low temperature is at least partially compensated for by a significantly higher rate of SR Ca^{2+} uptake in hibernating species as seen in Richardson's ground squirrels. As a consequence, the overall rate of SR Ca^{2+} uptake is still maintained at a relatively high level even at 5°C. Although there has been no direct measurement of Na^+-Ca^{2+} exchange activity in hibernating species, it may be expected that its activity at low temperature is high enough to extrude the Ca^{2+} that has entered the cell through Ca^{2+} channels and passive pathways because of the regular muscle contractions. This is confirmed in our present study (Chapter III). When Ca^{2+} extrusion via Na^+-Ca^{2+} exchange is decreased at 5°C by a reduction of extracellular Na^+, the ventricular muscles of Richardson's ground squirrels develop arrhythmic aftercontractions with an increase in resting tension and $[Ca^{2+}]_i$, which are similar to those of non-hibernating species treated with low temperature alone. Although sarcolemmal Ca^{2+}-ATPase has been studied in cardiac cells for over a decade, there is very little information about its temperature sensitivity. When such information becomes available, the contribution of sarcolemmal Ca^{2+}-ATPase to the regulation of $[Ca^{2+}]_i$ at low temperature may be evaluated.

5. Hypothermia-induced Positive Inotropy.

It is well known that low temperature induces an increase in cardiac contraction even when the myocardium is stimulated at a constant rate (Blinks and Koch-Weser 1963). The optimal temperature for maximal cardiac contraction is 25-20°C in non-hibernators and 15-10°C in hibernators (see Introduction). Three possible mechanisms may be responsible for this hypothermia-induced positive inotropy: 1) an increased Ca^{2+} influx across the sarcolemmal membrane; 2) increased Ca^{2+} release from the SR; and 3) increased Ca^{2+} sensitivity of the myofilaments.

An increase in peak Ca^{2+} current (I_{Ca}) through the voltage-dependent Ca^{2+} channels upon cooling has been observed in bullfrog cardiac muscle, which may account for the increase in cardiac contraction at low temperature in this species (Goto et al. 1978). In the mammalian hibernating species, the European hedgehog, the peak I_{Ca} does not change significantly in ventricular muscles when temperature is decreased from 35 to 10°C (Liu et al. 1991a). The inactivation of I_{Ca} is slowed at low temperature which allows more Ca^{2+} entry into the cell. However, the peak I_{Ca} of ventricular myocytes is decreased at low temperature in another hibernating species, the Richardson's ground squirrel (Herve et al. 1992). A marked decrease in cardiac peak I_{Ca} upon cooling has also been observed in many non-hibernators, including rat (Mitchell et al. 1983; Liu et al. 1991a), rabbit (Briggs and Bers 1991) and guinea pig (Cavalie et al. 1985; Herve et al. 1992). Thus, it seems that the Ca^{2+} influx through the voltage-dependent Ca^{2+} channels may not be the major source for hypothermia-induced positive inotropy in many mammals.

In a recent study, Ca^{2+}-release channels isolated from sheep cardiac SR have been incorporated into planar phospholipid bilayers. When temperature is decreased from 32 to 5°C, the single channel conductance is reduced linearly with a Q_{10} of 1.5 between 20 and 10°C. However, the open probability of the channel is increased upon cooling from 0.13 at 23°C to 0.69 at 10-5°C. As a consequence, the net effect of low temperature is to increase Ca^{2+} current through the SR Ca^{2+}-release channels (Sitsapesan et al. 1991). This indicates a contribution of increased Ca^{2+} release from SR to hypothermia-induced positive inotropy. However, the Ca^{2+} from SR may not be the only source. Even when SR Ca^{2+} release is inhibited by ryanodine, a specific SR Ca^{2+}-release channel blocker, there is still an increase in cardiac muscle contraction at low temperature although to a less degree (Shattock and Bers 1987). Since intracellular Na^{+} increases markedly as well at low temperature (Chapman 1986; Navas et al. 1990) and the sarcolemmal membrane is depolarized by cooling (Webb et al.

1969; Chapman 1986; Liu et al. 1991a), Shattock and Bers (1987) have suggested that Ca^{2+} influx via Na^+-Ca^{2+} exchange may be the major source for hypothermia-induced positive inotropy. However, recent studies (see above discussion) on temperature sensitivity, in terms of the Q_{10}, of Na^+-Ca^{2+} exchange do not support this hypothesis. On the other hand, reduced Ca^{2+} extrusion via Na^+-Ca^{2+} exchange, which is highly sensitive to low temperature in non-hibernators, will result in an increase in $[Ca^{2+}]_i$ and therefore muscle contraction.

An increase in the Ca^{2+} sensitivity of myofilaments may also account for hypothermia-induced positive inotropy. This has been observed in skinned skeletal muscles (Godt and Lindley 1982; Stephenson and Williams 1985) and canine Purkinje fibers (Fabiato 1985b). However, the myofilament Ca^{2+} sensitivity decreases markedly upon cooling in skinned cardiac ventricular muscles of rat, rabbit and frog (Harrison and Bers 1989; Harrison and Bers 1990a). Similar decrease in myofilament Ca^{2+} sensitivity upon cooling has also been observed in skinned ventricular muscles of a hibernating species, the Richardson's ground squirrel (Liu et al. 1993; Chapter V). These changes in myofilament Ca^{2+} sensitivity are due to the temperature effect on troponin C in different muscle fibers. When the troponin C of skinned cardiac muscle is replaced by the counterpart isolated from skeletal muscle, the cooling-induced shift in myofilament Ca^{2+} sensitivity is significantly reduced (Harrison and Bers 1990b). The main drawback of skinned muscle preparation is the loss of cytosolic soluble molecules which may affect the myofilament Ca^{2+} sensitivity at different temperatures. A recent study in perfused intact heart has indicated that positive inotropy in mild hypothermia (from 38 to 30°C) is partially due to an increase in maximal Ca^{2+}-activated force (Kusuoka et al. 1991). Therefore, the Ca^{2+} sensitivity of myofilaments at low temperature needs to be studied more thoroughly in intact muscles.

6. Membrane Fluidity and Cell Functions.

It is well accepted that biological membranes are fluid structures (Singer and Nicolson 1972). The kinetic and thermal responses of membrane-associated enzymes and proteins depend to a large extent on the physical state of the lipids which constitute the membrane bilayer. Many membrane functions have been correlated with membrane fluidity, which presumably changes through alterations in fatty acid unsaturation and cholesterol content (Deuticke and Haest 1987). For instance, the activity of SR Ca^{2+}-ATPase is directly modulated by lipid fluidity (Squier et al. 1988). Since the transition of membrane lipids from liquid-crystalline state to gel state can be induced by temperature, lowering temperature can influence membrane function either directly by acting on membrane enzymes and proteins, or by altering the lipid fluidity, or both.

Studies have indicated that the thermal transition and change of molecular organization of membrane lipids at low temperature are different between hibernating and non-hibernating species. In the sarcolemmal membrane of dog heart, there are two thermal transitions: one at 26°C and the other at 15°C (Raison et al. 1981). As a comparison, only one transition at 26°C is observed in summer active ground squirrels. Since 15°C is only a few degrees below the critical temperature for cardiac arrest of dog heart, it has been suggested that the thermal transition at 15°C may be associated with dysfunction of dog heart. When cardiac mitochondrial membrane is examined in another hibernating species, the thirteen-lined ground squirrel, no thermal transition is observed unless the temperature is lower than 4°C no matter the animal is in summer-active or winter-hibernating state (Raison et al. 1988). Evidence from research on mammalian hibernation has indicated that when an animal enters into hibernation, the degree of unsaturation of fatty acids increases and thus the fluid state of cardiac membranes is thought to be preserved at low temperature (Aloia et al. 1974; Raison et al. 1981).

It seems that the degree of unsaturation of membrane lipids is correlated to the cold tolerance of cardiac membrane and therefore cell function. In studies of the 1960-70's, the cold tolerance of the heart has been investigated under different diets in non-hibernating species. Following a diet of unsaturated fat for three months, the lethal temperature below which the rat heart stops beating is decreased from 12.9°C in control to 7.2°C (Huttunen and Johansson 1963). A further study on rat diet has shown that a highly unsaturated fat diet decreases the transition temperature at which discontinuity of succinate oxidation by myocardial homogenates occurs, and the temperature below which heart rate becomes irregular (Schatte et al. 1977). However, there is no change in the relationship of heart rate versus body temperature nor the ability to survive after profound hypothermia (Schatte et al. 1977).

Recent studies have indicated that low temperature has a direct inhibitory effect on membrane proteins. The isolated Na^+-Ca^{2+} exchanger proteins from dog and rabbit heart have been reconstituted into artificial lipid vesicles. Different lipid environments consisting of either asolectin or phosphatidylserine, phosphatidylcholine, and cholesterol have little effect on the temperature sensitivity of the Na^+-Ca^{2+} exchanger (Bersohn et al. 1991). The same results have also been observed in two poikilotherms, frog and trout (Bersohn et al. 1991; Tibbits et al. 1992). These results lead the authors to conclude that the temperature-sensitivity of cardiac Na^+-Ca^{2+} exchange is an intrinsic property of the protein, and is not dependent on species differences in membrane lipid composition.

7. $[Ca^{2+}]_i$ Homeostasis and Hypothermic Heart Preservation.

The present study has clearly demonstrated that cardiac $[Ca^{2+}]_i$ increases significantly at low temperature in non-hibernating species. The total cellular Ca^{2+} in cardiac cells also increases markedly upon cooling (Navas et al. 1990). Since abnormal increase in $[Ca^{2+}]_i$ impairs cellular metabolism and membrane integrity as discussed above, the management of $[Ca^{2+}]_i$ at low temperature is an important factor in functional recovery after rewarming. This is quite relevant to research on hypothermic heart preservation. Since both SR Ca^{2+}-ATPase activity and Na^+-Ca^{2+} exchange in the sarcolemmal membrane are markedly inhibited by low temperature in non-hibernating species, two Ca^{2+} sources may be responsible for the abnormal increase in $[Ca^{2+}]_i$ at low temperature. One is Ca^{2+} influx across the sarcolemmal membrane; the other is Ca^{2+} leakage from the SR. Many cardioplegic solutions employ zero Ca^{2+} to eliminate extracellular Ca^{2+}; however, this may not be effective in reducing SR Ca^{2+} because of the short period of the cold flush commonly used in the harvest of donor hearts (Wheeldon et al. 1992). Preliminary data from the present study indicate that a reduction of cellular Ca^{2+} loading before the heart is cooled for preservation improves recovery of cardiac function after rewarming. This suggests that management of $[Ca^{2+}]_i$ needs to be considered systematically in hypothermic heart preservation.

8. Conclusions.

In the present study, the effects of low temperature on cardiac $[Ca^{2+}]_i$ and different aspects of excitation-contraction coupling have been examined, and compared between hibernating and non-hibernating mammals.

The significant increase in cardiac $[Ca^{2+}]_i$ at low temperature observed in non-hibernating species is consistent with the hypothesis that Ca^{2+} overload, induced by cooling, is one of the main causes of severe arrhythmia in accidental hypothermia. Maintenance of normal contractile function at low temperature requires a superior ability to regulate $[Ca^{2+}]_i$. In hibernating species, this is closely related to the activities of SR Ca^{2+}-ATPase for SR Ca^{2+} uptake and the Na^+-Ca^{2+} exchanger for extrusion of Ca^{2+} from the cell.

Not only does low temperature result in a reduction of the Ca^{2+} influx through voltage-dependent Ca^{2+} channels, as demonstrated in previous studies (except the hedgehog heart); but the effect of organic Ca^{2+} channel blockers on muscle contraction is also decreased at low temperature. This is due either to a direct effect of cooling, or to a use-dependent property of the Ca^{2+} channel blockers. Therefore, when Ca^{2+} channel blockers are used to evaluate the role of Ca^{2+} channels on control of $[Ca^{2+}]_i$, caution should be used because of the above changes. Increased cardiac sensitivity to Cd^{2+} at low temperature needs to be further investigated.

Reduced Ca^{2+} sensitivity of myofilaments in both hibernating and nonhibernating species suggests that, at least in chemically-skinned cardiac muscles, hypothermia-induced positive inotropy of cardiac muscles is not due to increased myofilament Ca^{2+} sensitivity. Since Ca^{2+} influx is also markedly reduced upon cooling as observed in previous studies, hypothermia-induced positive inotropy is probably due to increased release of Ca^{2+} from the SR.

Since abnormal increase in $[Ca^{2+}]_i$ impairs cellular metabolism and induces cell injury, management of $[Ca^{2+}]_i$ at low temperature needs to be seriously considered in hypothermic heart preservation. Preliminary data from the present studies suggest that reduction in cellular Ca^{2+} before cooling has some beneficial effects on functional recovery following hypothermic preservation.

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Wier, W. G., Cannell, M. B., Berlin, J. R. Marban, E. and Lederer, W. J. (1987) Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by Fura-2. *Science Wash. DC* 235, 325-328.

VITA

GENERAL

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Sex:	Male
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AWARDS RECEIVED

April 1993 the Andrew Stewart Memorial Graduate Prize in the University of Alberta.

September 1989 - August 1991 a Studentship from the Alberta Heritage Foundation for Medical Research.

EDUCATION AND TRAINING

September 1988 - August 1993

Ph.D. degree in 1993.
Department of Zoology, University of Alberta, Edmonton, Alberta, Canada.
Thesis: Cardiac function and regulation of intracellular Ca^{2+} at low temperature in hibernating and non-hibernating mammals.
Supervisor: Dr. Lawrence C. H. Wang

March 1988 - June 1988

Research assistant in Department of Pharmacology, University of Essen, Germany.
Projects: (1). Activation of α-receptors and membrane ionic currents in single cardiac cells.
(2). Na^+-Ca^{2+} exchange current in single cardiac myocytes.
(3). Mechanical restitution in single cardiac cells.
Supervisor: Dr. Ursula Ravens

January 1987 - February 1988

Research assistant in Department of Pharmacology, University of Lund, Sweden.
Projects: (1). Temperature effects on the cardiac action potentials and membrane ion channels in mammalian hibernators and non-hibernators.
(2). Intracellular Ca^{2+} and the cardiac contractility in mammalian hibernators and non-hibernators.
(3). Mechanical restitution in the cardiac muscle from mammalian hibernators and non-hibernators.
Supervisors: Dr. Bengt W. Johansson
Dr. Björn Wohlfart

September 1985 - December 1986

Ph.D. student.
Department of Biology, Beijing University (Peking University).
Supervisor: Dr. Iping Chao (Yibing Zhao)

September 1982 - August 1985

M. Sc. degree in 1985.
Department of Biology, Beijing University (Peking University).
Thesis: Response of myocardial transmembrane potential of the hedgehog to temperature changes and the factors influencing it.
Supervisors: Dr. Iping Chao (Yibing Zhao)
Dr. Mingji Zhao

September 1978 - August 1982

B. Sc. degree in 1982.
Department of Biology, Beijing University (Peking University).
Thesis: Sensitivity and response to acetylcholine in sartorius and rectus abdominis muscles of toad.
Supervisor: Dr. Zuoqiang Huang

TEACHING EXPERIENCE

September 1992 - April 1993	Lab teaching in a course of Animal Biology.
September 1991 - April 1992	Lab teaching in a course of Animal Biology.
September 1988 - April 1989	Lab teaching in a course of Animal Biology.

PUBLICATIONS

(1). Liu, B. and Wang, L. C. H. (1993)
Regulation of cardiac cytosolic free Ca^{2+} at low temperature in the Richardson's ground squirrel. In: *Life in the Cold III: Ecological, Physiological, and Molecular Mechanisms.* eds. Carey, C., Florant, G. L., Wunder, B. A. and Horwitz, B. Westview Press, in press, (accepted on Feb.3, 1993).

(2). Liu, B., Wang, L. C. H. and Belke, D. D. (1993)
Effects of temperature and pH on cardiac myofilament Ca^{2+} sensitivity from rat and ground squirrel. *Am. J. Physiol.* 264, R104-R108.

(3). Liu, B., Belke, D. D., Jourdan, M. L. and Wang, L. C. H. (1992)
Cardiac function and myocardial calcium regulation at varying body temperatures. In: *Thermoregulation: The Pathophysiological Basis of Clinical Disorders.* eds. Lomax, P. and Schonbaum, E., pp. 109-113, Karger, Basel.

(4). Liu, B., Wang, L. C. H. and Belke, D. D. (1991)
Effect of low temperature on the cytosolic free Ca^{2+} in rat ventricular myocytes. *Cell Calcium* 12, 11-18.

(5). Zhou, Z.-Q., Liu, B., Dryden, W. F. and Wang, L. C. H. (1991)
Cardiac mechanical restitution in active and hibernating Richardson's ground squirrel. *Am. J. Physiol.* 260, R353-R358.

(6). Liu, B., Belke, D. D., Jourdan, M. L. and Wang, L. C. H. (1991)
Cardiac function and myocardial calcium regulation at varying body temperatures. In: *Pharmacology of Thermoregulation: Eighth International Symposium.* Kananaskis Village, Alberta, Canada, August 26-30, p41.

(7). Liu, B. Arlock, P., Wohlfart, B. and Johansson, B. W. (1991)
Temperature effects on the Na^+ and Ca^{2+} currents in rat and hedgehog ventricular muscle. *Cryobiology* 28, 96-104.

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Mechanical restitution at different temperatures in papillary muscles from rabbit, rat and hedgehog. *Cryobiology* 27, 596-604.

(9). Liu, B., Wohlfart, B. and Johansson, B. W. (1990b)
Effects of low temperature on contraction in papillary muscles from rabbit, rat and hedgehog. *Cryobiology* 27, 539-546.

(10). Zhou, Z.-Q., Liu, B., Dryden, W. F. and Wang, L. C. H. (1989)
The cardiac mechanical restitution in active and hibernating Richardson's ground squirrel (*Spermophilus richardsonii*). In: *Living in the Cold: International Symposium at Le Hohwald, France, April* 23-29, p77.

(11). Liu, B., Belke, D. D. and Wang, L. C. H. (1989)
Effect of prolonged cold exposure on cardiac post-rest contraction of rat and Richardson's ground squirrel (*Spermophilus richardsonii*). In: *Living in the Cold: International Symposium at Le Hohwald, France, April 23-29*, p42.

(12). Arlock, P., Liu, B., Wohlfart, B. and Johansson, B. W. (1988)
A comparison of some force-interval relations in rat, hedgehog, and human cardiac preparations. *Cardiovasc. Res.* 22, p601.

(13). Wohlfart, B., Arlock, P., Liu, B. and Johansson, B. W. (1988)
Excitation and contraction in myocardium: factors influencing peak force. *Cardiovasc. Res.* 22, 598-599.

(14). Liu, B., Ravens, U., Wang, X.-L. and Wettwer, E. (1988a)
Effects of α-adrenoceptor stimulation on membrane currents of ventricular myocytes of the rat heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 338, Supplement, R38.

(15). Liu, B., Ravens, U., Wang, X.-L. and Wettwer, E. (1988b)
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(16). Liu, B., Zhao, M. and Chao, I. (1987)
Effect of cold on transmembrane potentials in cardiac cells of the hedgehog. *J. Therm. Biol.* 12, 77-80.

(17). Liu, B. and Zhao, M. (1985)
Response of myocardial transmembrane potential of the hedgehog to temperature changes and the factors influencing it. *Proceedings of Chinese Physiological Society 1985 Conference.* 205-206.